10/681,479

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/71027 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/EP01/03311

(22) International Filing Date: 23 March 2001 (23.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00106450.0 24 March 2000 (24.03.2000) EP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: mRNA AMPLIFICATION

(57) Abstract: The present invention relates to a method for the amplification of mRNA of a sample, comprising the steps of i.) generating cDNA from polyadenylated RNA employing at least one primer hybridizing to said polyadenylated RNA and comprising a 5' poly(C) or a 5' poly(G) flank; ii.)(aa) if present, removing non-hybridized, surplus primer(s) and/or surplus dNTPs; ii.)(ab) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was employed; or ii.)(b) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was employed or a poly(C) tail when in step i. (a) primer(s) comprisng a 5' poly(G) flank was employed using an RNA-ligase, irrespective of the presence or absence of surplus primer(s) and/or surplus dNTPs; and iii.) amplifying the tailed cDNA with a primer hybridizing to the tail(s) generated in step ii(ab) or ii(b). Furthermore, the present invention relates to methods for the preparation of in vitro surrogate(s), for identifying expressed genes in a test sample, for identifying a drug candidate for therapy of a pathological condition and for in vitro detection of a pathological condition employing said method for amplification of mRNA. In addition, the present invention relates to the use of amplified cDNA(s) as obtained by the method of the invention in hybridization, interaction and/or enzymatic arrays.

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mRNA amplification

The present invention relates to a method for the amplification of mRNA of a sample, comprising the steps of i.) generating cDNA from polyadenylated RNA employing at least one primer hybridizing to said polyadenylated RNA and comprising a 5' poly(C) or a 5' poly(G) flank; ii.)(aa) if present, removing non-hybridized, surplus primer(s) and/or surplus dNTPs; ii.)(ab) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was employed; or ii.)(b) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was employed using an RNA-ligase, irrespective of the presence or absence of surplus primer(s) and/or surplus dNTPs; and iii.) amplifying the tailed cDNA with a primer hybridizing to the tail(s) generated in step ii(ab) or ii(b). Furthermore, the present invention relates to methods for the preparation of in vitro surrogate(s), for identifying expressed genes in a test sample, for identifying a drug candidate for therapy of a pathological condition and for in vitro detection of a pathological condition employing said method for amplification of mRNA. In addition, the present invention relates to the use of amplified cDNA(s) as obtained by the method of the invention in hybridization, interaction and/or enzymatic arrays.

Several documents are cited throughout the text of this specification. The disclosure content of each of the documents (including any manufacturer's specifications, instructions, etc.) is herewith incorporated by reference.

The study of gene expression and gene expression patterns have lately been revolutionized by global analysis of mRNA expression on cDNA filter assays or cDNA micro arrays (see, inter alia, Southern, Trends Genet. 12 (1996), 110-115;Debouck, Nat. Genet. 21:48-50 (1999); Hacia, Nat. Genet., 21, 42-7 (1999); Cole, Nat. Genet. 21, 38-

41 (1999); Bowtell DD., Nat. Genet., 21, 25-32 (1999); Cheung, Nat. Genet., 21, 15-19 (1999); Duggan, Nat. Genet., 21, 10-14 (1999); Southern, Nat. Genet., 21, 5-9 (1999)). For example, Lockhart (Nature Biotechnology 14 (1996), 1675-1680) describes an approach that is based on hybridization of a large number of mRNAs to small, high-density arrays containing tens of thousands of synthetic oligonucleotides, allowing for the simultaneous monitoring of tens of thousands of (expressed) genes. Further micro arrays for gene expression have been described in Shalon (Pathol. Biol. 46 (1998), 107-109), Lockhardt (Nuc. Acids Symp. Ser. 38 (1998), 11-12) or in Schena (Trends Biotech. 16 (1998), 301-306). However, one of the major draw-backs of the above described cDNA-array technology is the fact that these technologies require an amount of 2.5 to 10 µg of nucleic acid probes to be tested either in the form of mRNA, reverse transcribed RNA or amplified cDNA (see, inter alia, Schena (Science 270 (1995), 467-470 and PNAS U.S.A. 93 (1996), 10614-10619) or Lockhardt (1996) loc. cit.). This amount of material is normally only derived from a large of number of cells such as about 10^9. Bryant, PNAS U.S.A. 96 (1999), 5559-5564 or Mahadevappa, Nat. Biotech. 17 (1999), 1134-1136 reported such an approach using at least from 50000 cells. The smallest number of cells yet used for ex-vivo tissue analysis and corresponding gene expression has been 1,000 cells (Luo, Nat. Medicine 5 (1999), 117-122). However, a plethora of physiological and/or pathological conditions would require to study the gene expression pattern or "transcriptome", defined as the entirety of mRNA molecules in a given biological sample (Velculescu, Cell, 88, 243-251 (1997) of a lower number of cells or even a single cell. For instance, the investigation of spatially and temporally regulated gene expression in embryogenesis would clearly profit from a method were a low number of cells, in particular a single cell, can be deduced. Similarly, it would be of high interest to investigate the gene expression pattern/transcriptome of individual cells or a low number of cells derived from adult tissue, like, inter alia, blood or neuronal (stem) cells. Furthermore, multiple pathological conditions could be clarified, e.g., the delineation of deregulated gene expression in atypical proliferation, mutaplasia, preneoplastic lesians and/or carcinomata in situ. Other examples of locally restricted pathological processes which could be investigated comprise, but are not limited to, restenosis, Alzheimer's disease, Parkinson's disease, graft-versus-host disease or inflammations in autoimmunity. Furthermore, occult micrometastasis derived from a

small cancer has dire consequences if the disseminated tumor cells survive in distant organs and grow into manifest metastases. Tumor cells left after resection of primary tumors are currently detected in bone marrow aspirates by immunocytochemical staining with antibodies directed against cytokeratins (reviewed in Pantel, J. Natl. Canc. Inst. 91, 1113-1124 (1999)). While several studies have established the prognostic significance of cytokeratin-positive micrometastatic cells in bone marrow (Braun, N. Engl. J. Med. 342, 525-533 (2000); Pantel, J. Natl. Canc. Inst. 91, 1113-1124 (1999)), the biology of these cells has largely remained enigmatic because of their extremely low frequency in the range of 10^{-5} -10^{-6}.

The systemic spread of cancer cells requires that cells evade from the solid tumor, distribute via blood or lymphatic vessels, cross endothelial and tissue barriers and survive ectopically as single cells. The phenotypic changes accompanying these steps are considered a developmental process, the so-called epithelial-mesenchymal transformation (EMT) (Hay, Acta Anatomica, 154, 8-20, (1995); Birchmeier, Acta Anatomica, 156, 217-226 (1996)). Only a small fraction of cells disseminated from a tumor may acquire EMT-associated features (Boyer, Acta Anatomica, 156, 227-239 (1996)). The epigenetic changes leading to EMT are not known so far but may have important implications for the development of future therapies.

Major technical hurdles in studying epigenetic changes of, e.g., disseminated tumor cells or pathological modified tissue are limited accessibility, low frequency, unambiguous identification, and subsequent transcriptome analysis at a single cell level or of a low number of cells. A variety of protocols has been developed for the generation of "single cell cDNA libraries" and the global amplification of mRNA from individual cells (see Belyavsky, Nucl. Acid. Res., 17, 2919-2932 (1989); Brady, Methods in Enzymology, 225, 611-623 (1993); and Karrer, Proc. Natl. Acad. Sci. USA, 92, 3814-3818 (1995)). However, these procedures have obvious drawbacks, such as the restriction to 3'-ends and an insufficient sensitivity when PCR amplificates are hybridized to cDNA arrays.

In these procedures, variation introduced during amplification of cDNA fragments was reduced by limiting the length of the cDNAs during reverse-transcription. This was

accomplished through low substrate conditions for the reverse-transcriptase; i.e. the use of low concentrations of an oligo d(T) primer and low dNTP concentrations. However, there is a risk of compromising reverse-transcription and subsequent PCR-efficiency which may lead to arbitrary results when transcriptome/gene expression patterns of cells/single cells are to be investigated. Furthermore, the use of an oligo(dT) primer for PCR amplification limits the use of high annealing temperatures and thus stringent annealing conditions. Typically, annealing is performed at 42°C (Brail, Mut. Res. Genomics 406 (1999), 45–54). As pointed out hereinabove, such an approach may be suitable for a 3' restricted cDNA synthesis. However, higher annealing temperatures reduce the presence of secondary structures in the cDNA and the likelihood of unspecific annealing to internal sequences of the cDNA, which would result in shortening of the amplificates compared to the cDNA molecules. Annealing temperatures of the method of the invention are preferably above 45°C, more preferably above 55°C, even more preferably above 65°C.

As mentioned hereinabove, the amount of mRNA in a low number of cells or even a single cell is insufficient for use in direct global analysis. Therefore, global analysis of expressed mRNA (of a "transcriptome") from a low number of cells or even an individual, single cell requires amplification of extracted and/or reverse transcribed polyadenylated mRNA. To date, PCR amplification of small amounts of mRNA has not resulted in reliable representation of the relative expression of mRNA present in a certain cell/low number of cells at a specific timepoint, a specific developmental state and/or a specific physiological state (Brail, Mut. Res. Genomics 406 (1999), 45–54), Brail (1999), (loc. cit.) conclude that the method as described by Brady (Brady (1993) (loc. cit.) is likely to introduce variation(s) in the tailing reaction or the PCR amplification steps. In particular, Brail's analysis (Brail (1999), loc. cit) showed a five-fold variation even for highly-abundant house-keeping genes (direct comparison of GAPDH and ribosomal gene L32).

Thus, the technical problem of the present invention consists in providing means and methods which comply with the need of a global and uniform amplification of mRNA, in particular of the transcriptome of a low number of cells or a single cell. The solution to

this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, present invention relates to a method for amplification of mRNA of a sample, comprising the steps of

(i) generating cDNA from polyadenylated RNA employing at least one primer hybridizing to said polyadenylated RNA and comprising a 5' poly(C) or a 5' poly(G) flank;

(ii)(aa) if present, removing non-hybridized, surplus primer(s) and/or surplus dNTPs;

(ab) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed; or

(b) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed using an RNA-ligase, irrespective of the presence or absence of surplus primer(s) and/or surplus dNTPs; and

(iii) amplifying the tailed cDNA with a primer hybridizing to the tail(s) generated in step (ii)(ab) or (ii)(b).

Polyadenylated RNA can be obtained from a sample by methods known in the art. These methods comprise oligo (dT) selection steps. The sample may be of animal or plant origin and may be a tissue or a cell sample. Said sample may also comprise cultured tissue(s) or cultured cell(s). Particularly preferred is a sample of human origin. Samples may be obtained by methods known in the art, which comprise, but are not limited to atherectomy, debulking, biopsy, laser dissection or macroscopic surgical procedures.

The here described technique and method for amplification of mRNA from said sample comprises steps, wherein said polyadenylated RNA obtained from a sample is employed for the generation of (a) first cDNA product(s) employing (a) primer(s) comprising 5'-oligo (dC)/poly (C) (-or 5'-oligo (dG)/poly (G)) flanking regions. Said 5'-

oligo (dC) or 5'-oligo (dG) primer preferably comprises between 8 and 20 cytosine (or guanine) nucleotides, more preferably 10 cytosine (or guanine) nucleotides, more preferably said primer(s) comprise(s) 11, even more preferably said primer(s) comprise(s) 13, most preferably said primer(s) comprise(s) 15 cytosine (or guanine) nucleotides. It is preferred that the first cDNA synthesis is carried out after potentially contaminating tRNAs or rRNAs have been removed. Such a removal can be carried out by methods known to the skilled artisan, for example, by binding the polyadenylated mRNA to oligo (dT)/poly(T)-coated solid supports as defined herein and subsequent washing steps.

Furthermore, this first cDNA synthesis step comprises preferably random primers which are present in a concentration which is 2,000 to 8,000 times higher than primer concentrations used in previous studies (for example, of 10 nM as employed in Trumper, Blood 81 (1993), 3097-3115). It is furthermore preferred that said first cDNA synthesis, i.e. the generation of cDNA from polyadenylated RNA, is carried out in a correspondingly high concentration of dNTPs, preferably in a concentration of 0.5 mM dNTPs. This first cDNA preparation step (step "i") may also comprise means for labeling the resulting cDNA. Labels may be introduced by methods known to the skilled artisan (see, inter alia, "Current Protocols in Molecular Biology", edited by Ausubel et al., John Wiley & Sons, USA (1988)), and may comprise the employment of labeled dNTPs (like biotin-labeled, radio-labeled or fluorescein-labeled dNTPs). This first cDNA synthesis step (reverse transcription), employing preferably randomized primers, may comprise the use of standard enzymes, preferably RNAse H deficient reverse transcriptase, like Superscript II Reverse Transcriptase (GIBCO).

Since high dNTP concentrations improve said first cDNA synthesis but may interfere with any subsequent reactions (like tailing reactions) it is preferred that (before carrying out any further reactions and/or steps of the method of the invention) free surplus dNTPs are removed. Surplus, non-hybridized primer(s) are preferably also removed before additional steps are carried out. Said removal can be obtained, inter alia, by washing steps, like buffer exchanges (as shown in the appended examples), or by filtration methods (i.e. over size-selective membranes). However, said removal step can also be omitted should no surplus of dNTPs and/or primers be present. Furthermore,

the removal step can be avoided, should the subsequent "tailing-step" be carried out by an RNA-ligase step.

The 3'-tailing reaction of the method of the present invention (see step (ii)(ab) or (ii)(b) of the method of the invention) comprises the tailing with poly(G) when in step "i" (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) when in step "i" (a) primer(s) comprising a 5' poly(G) flank was/were employed. As demonstrated in the appended examples, it has surprisingly been found that, inter alia, poly(C) primers binding to poly(G)-tails are at least 100-times more sensitive than poly (T) primers binding on poly(A) tails, as proposed in the prior art (Brady (1993), loc. cit.; Trumper, Blood, 81, 3097-3115 (1993)).

The tailing reaction may be carried out by employing an enzyme with 3' terminal deoxynucleotide transferase activity, preferably in a non-cacodylate containing storage buffer, like terminal deoxynucleotide transferase (MBI Fermentas; Pharmacia) However, it should be mentioned that said "tailing"-step can also be carried out by RNA-ligase (see: Edwards, Nucl. Acids Res., 19, 5227-5232 (1991)). In this case, oligo(dC) or oligo(dG) flanking regions may be ligated to the 3-end of the single-stranded cDNA molecules by said RNA ligase. Sequences of the flanking regions are capable of hybridizing to the flanking region of the cDNA synthesis primer(s), (Edwards, Nucl. Acid Res. 19 (1991), 5227-5232).

Finally, the polyG/polyC-tailed cDNA can be further amplified since these cDNA(s) comprise(s) a 5' primer-introduced oligo(C) (or-G) stretch and a 3' oligo(G) (or-C) stretch introduced by, e.g., terminal deoxynucleotide transferase. This second PCR reaction may be carried out in the presence of labeled nucleotides. Preferred are biotin-labeled, fluorescein-labeled, dioxygenin-labeled or radio-labeled nucleotides which are known in the art. Furthermore, it is within the scope of this invention that "tagged" oligonucleotide primers (like biotin-, fluorescein-, dioxygenin-, or radio-labeled oligonucleotide primers.) are employed in order to obtain a single tag/label per cDNA species.

In a preferred embodiment of the method of the invention, said at least one primer in step "i" is a random primer, a oligo(dT) primer or a combination thereof. Said random primer may comprise a stretch of 4 to 10 random nucleotides, preferably a stretch of 5

to 9 random nucleotides. Most preferably said random primer comprises a random hexamer or a random octamer oligonucleotide. It is particularly preferred that said random primer has a sequence as shown in SEQ ID NOs: 1-8. Even more particularly preferred is the random primer CFI5CN6, as employed in the appended examples comprising the nucleotides 5'-$(CCC)_5$GTCTAG-A$(N)_6$ (SEQ ID NO: 8).

As shown in the appended examples, said random primer(s) can also be employed in combination with other random primers or (an) oligo(dT) primer(s). For example, in step "i" of the present invention a primer pair (CFI5c8, corresponding to SEQ ID NO: 9) and (CFI5cT, corresponding to SEQ ID NO: 10) may be employed, comprising the sequences 5'-$(CCC)_5$GTCTAGA$(N)_8$ and 5'-$(CCC)_5$GTCTAGATT$(TTT)_4$TVN, wherein "V" represents G, C or A and N represents G, T, C or A. Therefore, it is particularly preferred that a combination of a poly d(C)/(G) primer comprising an octamer (see, e.g. SEQ ID NO: 9) is employed in combination with an oligo (dT) primer (see, SEQ ID NO: 10).

Accordingly, in a further preferred embodiment of the method of the present invention, the oligo(dT) primer to be employed in step "i" has the sequence as shown in SEQ ID NO: 10, comprising the sequence 5'-$(CCC)_5$GTCTAGATT$(TTT)_4$TVN . As mentioned, hereinabove, said oligo (dT) primer(s) to be employed in step "i" of the method of the present invention can be used alone or in combination with (a) random primer(s) as described hereinabove. Said oligo (dT) primer(s) is/are preferably a primer comprising an oligo (dT) stretch.

In another preferred embodiment of the method of the present invention, the concentration of said at least one primer in step "i" is in the range of 0.01 µM to 500 µM, preferably in the range of 0.1 µM to 200 µM, more preferably in the range of 1 µM to 100 µM, even more preferably in the range of 10 µM to 60 µM. As shown in the appended example, the most preferred concentration is about 50 µM.

In yet another preferred embodiment of the method of the present invention, said primer in step "iii" comprises a stretch of at least 10, preferably at least 12, most preferably at least 15 nucleotides capable of hybridizing with the tail(s) generated in step "ii(ab)" or

"ii(b)". It is preferred that said primer does not comprise more than 20 nucleotide capable of hybridizing with the tail(s) generated in step "ii(ab)" or "ii(b)" of the method of the present invention. In a preferred embodiment said primer in step "iii" has the sequence as depicted in SEQ ID NO: 11, 12, 13, 14 or 15. As shown in the appended examples a particular preferred primer in step "iii" is the primer "CP2" comprising the nucleotide sequence 5'TCAGAATTCATG$(CCC)_5$ (see SEQ ID NO: 14), with which particularly good results have been obtained in this "global amplification" step. Therefore, should a single primer be employed in this step, the above described "CP2"-primer is particularly preferred when in step "ii(ab)" or "ii(b)" a poly(G)-tailing was carried out. An advantage of employing only a single primer in step "iv" of the invention is that potential "primer-primer" interactions can be avoided and relatively high primer concentrations preferably above 0.2 µM, more preferably above 0.8 µM, even more preferably above 1,0 µM can be used. Higher primer concentrations above 1,0 µM or 1,2 µM may also be employed.

In another preferred embodiment of the method of the present invention, said polyadenylated RNA (and/or mRNA to be amplified) is bound to a solid support. Said solid support may be, inter alia, a bead, a membrane, a filter, a well, a chip or a tube. Particularly preferred is a magnetic bead, a latex bead or a colloid metal bead. However, said polyadenylated RNA may also be bound on solid supports like polystyrene beads. Solid phases known in the art also comprise glass and/or silicon surfaces, nitrocellulose strips or membranes and plastic (test) tubes. Suitable methods of immobilizing nucleic acids, in particular polyadenylated RNA on solid phases include but are not limited to ionic, hydrophobic, covalent interactions and the like. The solid phase can retain one or more additional receptor(s) like, for example, a poly (T) stretch, which has/have the ability to attract and immobilize the polyadenylated RNA. This receptor can also comprise a changed substance that is oppositely charged with respect to the nucleic acid. In a most preferred embodiment of the method of the present invention, the solid support, (like said magnetic bead) comprises therefore an oligo(dT) stretch.

As shown in the appended examples, the mRNA/polyadenylated RNA to be amplified by the method of the present invention can easily be isolated on an oligo (dT) coated solid support, like oligo (dT) coated magnetic beads.

In yet another embodiment of the present invention the mRNA to be amplified is derived from a tissue, a low number of cells or a single cell. Said low number of cells may be in the range of 10^6 to 2 cells. Said tissue, cells or single cell may be of plant or animal origin. It is particularly preferred that said tissue, cells or single cell is/are of human origin. Said tissue, cells or single cell may be, furthermore, a pathological sample and/or a sample which is suspected to be pathological. Whether pathological, suspected to be pathological or normal/healthy, said tissue, (low number of) cells or single cell may be derived from a body fluid or from solid tissue. Body fluids may comprise blood, lymphatic fluid, peritonal fluid, spinal/cerebrospinal fluid, amnionic fluid, urine or stool. Said solid tissue may be derived from all animal/human organs or glands. Furthermore, said tissue may comprise malignant transformations, like tumors or restenotic tissue. Therefore, said tissue, (low number of) cells, or single cells may also be from carcinomas, sarcomas, lymphomas, leukemias or gliomas. However, it should be pointed out that the method of the present invention can also be employed on samples derived from benign tissue, normal tissue as well as from cultured samples, like tissue and/or cell cultures. Tissues, low number of cells and/or single cells can be obtained by methods known in the art, which comprise, but are not limited to biopsis, aspirations or dilutions. Samples can also be separated and obtained by FACS sorting or isolation by immunological methods or "receptor/ligand" binding methods. As shown in the appended examples, samples can also be obtained by artherectomy, e.g. helical device for artherectomy (X-sizer, Endicor)

In another preferred embodiment of the method of the present invention, said tissue, low number of cells or single cell is a chemically fixed tissue, chemically fixed low number of cells or chemically fixed cell. Said fixation may be carried out in (para)formaldehyde. Preferred concentrations are in the range of 0.1 to 1%, most preferred is, however, a concentration of 0.1%. Said fixation is preferably carried out for

less than 30 minutes (when concentrations below 1% are employed). Most preferably said fixation is carried out at a (para)formaldehyde concentration of 0.1% for 5 minutes.

In another preferred embodiment, the method of the present invention further comprises a step "iv" wherein the generated amplified cDNA is further modified. Said modification may comprise the introduction of means for detection, for example, the introduction of nucleotide analogues coupled to (a) chromophore(s), (a) fluorescent dye(s), (a) radio-nucleotide(s), biotin or DIG. Labeling of amplificated cDNA can be performed as described in the appended examples or as described, inter alia, in Spirin (1999), Invest. Opthalmol. Vis. Sci. 40, 3108-3115.

Furthermore, it is preferred that the obtained amplified cDNA is bound to a solid support, as defined hereinabove.

Since standard cacodylate containing buffers (like some cDNA synthesis buffers) may interfere with individual steps of the method of the invention (like the "tailing reaction") it is preferred that all or individual steps are carried out in a non-cacodylate buffer. Particularly preferred is a phosphate buffer and most preferred is a KH_2PO_4 buffer as employed in the appended examples. Preferably said buffer is a buffer of low ionic strength (see Nelson, Methods in Enzymology, 68, 41-50 (1979)). Furthermore, the use of dGTP or dCTP in "tailing" reactions leads to short extension of 15 – 30 nucleotides, while the use of dATP or dTTP leads to long extensions ranging from 70 to several hundred nucleotides (Nelson (1979), loc. cit.; MBI Fermentas 1998/1999 catalog, p. 125); Deng, Methods Enzymology, 100, 96-116, (1983)). Long poly(dA)/(dT) tails, however, result in non-homogeneous populations of cDNAs during amplification due to various hybridization / annealing sites. In contrast, the method of the invention with its short (10-30 bases) 5' primer and 3'tailing introduced oligo(dC) or oligo(dG) flanking regions generate homogenous populations of amplified cDNAs, amplifying preferentially the coding regions of the original cDNA molecules.

In yet a more preferred embodiment of the method of the present invention, the sample comprising mRNA/polyadenylated RNA to be amplified is derived from a cell and/or a

tissue (or is a cell and/or a tissue), the genetic identity of which had been defined by comparative genomic hybridization (CGH). As shown in the appended examples, a method comprising CGH of a single cell (SCOMP; see Klein (1999), PNAS USA 96, 4494-4499)) has recently been described which allows for unambiguous identification of a single cell. With this method it is possible to identify, inter alia, a tumor cell and/or a cell of tumerous origin by its chromosomal aberrations. Employing the here described method for mRNA amplification and combining said method with SCOMP, it is therefore possible to isolate genomic DNA and mRNA from the same single cell.

The present invention also relates to a method for the preparation of an in vitro surrogate for (a) pathologically modified cell(s) or tissue(s) comprising the steps of:

(a) amplifying mRNA of said pathologically modified cell(s) or tissue(s) according to the steps of the method described herein above;

(b) assessing the quantity and, optionally, biophysical characteristics of the obtained cDNA and/or transcripts thereof, thereby determining the gene expression pattern of said pathologically modified cell(s) or tissue(s);

(c) selecting an in vitro cell, the gene expression pattern of which resembles the gene expression pattern of said pathologically modified cell(s) or tissue(s); and

(d) adapting the gene expression pattern of said in vitro cell to the gene expression pattern of the pathologically modified cell or tissue.

The term "in vitro surrogate" as used herein means (a) cell(s) or (a) cell line(s) which is capable of mimicking a pathological situation or a pathological condition. Said surrogate may be useful, inter alia, in medical, pharmacological or scientific experiments and may be employed for drug screening purposes. In particular, such a surrogate cell/cell line may be employed to identify potential drugs and/or medicaments. Such identification may be carried out by screening libraries of chemicals and/or biologics, and, preferably, said surrogate(s) is/are used in high throughput-screenings.

The assessment of the quantity and, optionally the biophysical characteristics of the obtained cDNA and/or transcripts thereof can be carried out by methods known to the person skilled in the art and/or as described herein.

The term "in vitro cell" as employed in accordance with this invention preferably relates to a cell which may be maintained in culture. Said cell is preferably maintained in culture for at least 1 hour, more preferably for at least 6 hours, more preferably for at least 12 hours, more preferably for at least one day, more preferably for at least two days, more preferably for at least 3 days, more preferably for at least one week, most preferably for several weeks.

It is particularly preferred that said surrogate/in vitro surrogate faithfully reflects the transcriptome/gene expression pattern of the pathologically modified cell or tissue.
Said surrogate should closely resemble the pathologically modified tissue or pathologically modified cell. It is therefore preferred that the "in vitro cell" as mentioned in step c. herein above is similar to the pathologically modified tissue/cell. For example, the "in vitro cell" may be derived from a similar tissue or organ as the pathologically modified/diseased tissue. Inter alia, coronary artery smooth muscle cells can be employed as "in vitro cells", the gene expression pattern of which resembles the gene expression pattern of restenotic tissue. Similar, liver cells (like, e.g, HepG2) may be employed to obtain a surrogate for pathologically modified liver tissue, cultured renal cells (like, e.g. ATCC 45505) for kidney diseased tissue, cardiomyoblasts (like, e.g., rat cardiomyocyte) for heart muscle diseased tissue, or NCI cell lines as described in Ross, Nat. Genetics 24 (2000), 227-235 for tumerous diseases, neoplastic diseases or cancer.

Said "adaption" of step (d) as mentioned herein above is carried out in order to adapt the gene expression pattern of the selected "in vitro cell" to a gene expression pattern which reflects more closely the gene expression pattern of the pathologically modified tissue/cell. In particular, when it was found (in steps (a) and (b) of the method as described herein above), that a particular transcript/expressed gene (or a group of particular transcripts/expressed genes) was downregulated in comparison to said "in vitro cell" (or a control cell), it should be attempted to upregulate the expression said transcript/expressed gene (or group of said transcripts/expressed genes) in said "in vitro cell". Accordingly, should a specific transcript/expressed gene (or a group of specific transcripts/expressed genes) be upregulated in comparison to said "in vitro cell" (or a

control cell), it should be attempted to downregulate said transcript/expressed gene (or a group thereof) in said "in vitro cell". Particular methods, factors, compounds and/or substances which may be useful to adapt the gene expression pattern of said in vitro cell are described herein below.

In one embodiment, it is preferred that said adaption step comprises contacting said in vitro cell with at least one compound, factor, substance, a plurality of compounds, factors, susbtances or a combination thereof and assessing whether said contacting leads to a modified gene expression pattern/transcriptome in said in vitro cell. The assessment of the gene expression pattern may be carried out by the method of the invention but may also comprise other analysis methods known in the art, like biochemical or biophysical methods. Particularly preferred are hereby methods like proteome analysis, comprising one- or two dimensional (gel) electrophoresis, high-performance liquid chromatography, mass spectrography or antibody-based detection methods (blotting or array systems).

The above mentioned pathologically modified cell(s) or tissue(s) an/or in vitro cell is preferably of animal origin. Particularly preferred are hereby cell(s) or tissue(s) derived and/or obtained from primates, rodents or artiodactyles. Even more preferred are cell(s) and/or tissue(s) from humans, monkeys, pigs, cows, rats or mice.

In yet another embodiment, the method for the preparation of an in vitro surrogate for (a) pathologically modified cell(s) or tissue(s) comprises the further steps of

b(1). determining the gene expression pattern of (a) control cell(s) or (a) control tissue(s); and

b(2). determing the gene(s) which is/are differentially expressed in said for pathologically modified cell(s) or tissue(s) and said control cell(s) or tissue(s).

The here mentioned control cell(s) or control tissue(s) can be easily determined by the person skilled in the art. For example, similar tissue from a healthy donor may be employed. As shown, e.g., in the appended examples a control tissue for restenotic tissue may be media or media/intima of healthy coronary arteries. Furthermore, control

cell(s) or control tissue(s) may be obtained during biopsis of hepatic tissue, renal tissue, prostate, cervical tissue etc.

It is particularly preferred that the gene expression pattern, i.e. the "transcriptome" of said control cell or control tissue is also determined by employing the method for the amplification of mRNA of a sample as described herein. Preferably, said transcriptome analysis of samples like the pathologically modified cell(s) or tissue(s), the control cell(s) or control tissue(s) comprises the steps of

i. generating cDNA from polyadenylated RNA of said pathologically modified cell or tissue, said control cell or tissue and/ or said in vitro cell employing at least one primer hybridizing to said polyadenylated RNA and comprising a 5' poly(C) or a 5' poly(G) flank;

ii.(aa) if present, removing non-hybridized, surplus primer(s) and/or surplus dNTPs;

(ab) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed; or

(b) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed using an RNA-ligase, irrespective of the presence or absence of surplus primer(s) and/or surplus dNTPs;

iii. amplifying the tailed cDNA with a primer hybridizing to the tail(s) generated in step ii(ab) or ii(b);

iv. employing the amplified cDNA in (a) hybridization assays; and

v. detecting differences and/or similarities in the gene expression pattern of said pathologically modified cell or tissue, said control cell or tissue and/ or said in vitro cell

The embodiments as described herein above for the method of the invention may be applied for said transcriptome analysis of said pathologically modified cell(s) or tissue(s), control cell(s) or control tissue(s) and/or said in vitro cell.

The above described method for the preparation of an in vitro surrogate can be, inter alia employed for restenotic tissue or for an restenotic cell. Said control cell or said control tissue(s) may be selected from the group consisting of smooth muscle cells, media/intima of (healthy) coronary arteries and media/intima of (healthy) peripheral arteries.

The "in vitro cell" to be accepted to the gene expression pattern of a pathologically modified cell(s) or tissue(s) may be derived from primary cell culture, a secondary cell culture, a tissue culture or a cell line. Preferably, these cells and/or cell cultures are, but are not limited to, cultured muscle cells, cultured smooth muscle cells, cultured coronary artery smooth muscle cells, HepG2 cells, Jurkat cells, THP-1 cells, Monomac-6 cells or U937-cells. Such cells are easily obtainable from sources known in the art, like DSMZ, Braunschweig or the ATCC, USA. Furthermore, cardiomyoblasts may be employed as "in vitro cell" for adaption to a "surrogate".

Said adaption step (step d. of the above described method for the preparation of an in vitro surrogate) may comprise the exposure of said in vitro cell to physical and/or chemical change(s), wherein said physical change(s) may comprise temperature shifts, light changes, pressure, pH-changes, changes in ionic strength or changes in the composition of gas phase(s) (like O_2, N_2, CO, CO_2) and said chemical changes may comprise medium exchanges, medium substitutions, medium depletions and/or medium additions. It is particularly preferred that said chemical changes comprise the exposure to compounds like growth factors, hormones, vitamines, antibodies or fragments and/or derivatives thereof, small molecule ligands, cytokines, transcription factors, kinases, antibiotics, natural and/or non-natural receptor ligands, or components of signal transduction pathways. Said adaptation step may also comprise co-culturing with other cells/cell lines, for example co-culturing with blood cells, glial cells, dendritic cells or osteoclasts. Said blood cell may comprise monocytes and T-lymphocytes.

In an even more preferred embodiment of the method for the preparation of an in vitro surrogate, said cytokine is IFN-γ (or a functional derivative therof), said natural and/or non-natural receptor ligand is a ligand for IFN-γ receptor (a and/or b chain), said

transcription factor is IRF-1 or ISGF3-γ-(p48), said kinase is tyrosine kinase Pyk2, said components of signal transduction pathways is Dap-1, BAG-1, Pim-1 or IFN-γ-inducible protein 9-27, said growth factor is platelet growth factor AA, angiotensin or fibroblast growth factor or said antibiotic is rapamycin.

In this context, the term "functional derivative" of IFN-γ relates to derivatives that retain or essentially retain the biological properties of natural IFN-γ. Examples of such derivatives are muteins. The same applies, mutatis mutandis, for other components mentioned herein.

In vitro surrogate(s) as obtained by the above described methods are particulary useful in drug screening methods and/or in toxicological analysis. Such methods comprise, but are not limited to the detection of modified gene expression pattern after contacting said in vitro surrogate with a test substance and/or a potential drug candidate. Such screening methods are well known in the art and are, inter alia, described in Scherf, Nat. Genetics 24 (2000), 236-244; Ross, Nat. Genetics 24 (2000), 227-235. High-throughput screenings are described and/or reviewed in Sundberg, Curr. Opin. Biotechnol. 11 (2000), 47-53; Hopfinger, Curr. Opin. Biotechnol. 11 (2000), 97-103; Vidal, Trends Biotechnol. 17 (1999), 374-381; Gonzales, Curr. Opin. Biotechnol. 9 (1989), 624-631; Fernandes, Curr. Opin. Chem. Biol. 2 (1998), 597-603.

Additionally, the present invention relates to a method for identifying differentially expressed genes in a test sample, wherein said method comprises the steps of (a) providing a test sample and a control sample each comprising polyadenylated RNA; (b) employing the steps of the method for the amplification of mRNA of the present invention on said test and control sample; and (c) comparing the obtained amplified cDNA of said test sample with the obtained amplified cDNA of said control sample. The test and control sample may be derived from the same organism but may also be derived from different organisms/individuals. Furthermore, said test sample may comprise tissue cultures or cell cultures. Furthermore, said test and/or control sample comprises preferably the same kind of cell(s) and/or tissue(s). The comparison of step (c) can be carried out as, for example, shown in the appended examples and may

involve hybridization of obtained amplified cDNA to cDNA arrays. The method for identifying differentially expressed genes may therefore comprise the comparison of tissue, (a low number of) cells or a single cell of distinct origin. For example, pathological and non-pathological tissue, (low number of) cells or single cells may be compared on the transcriptome level.

The present invention also relates to a method for identifying a drug candidate for prevention or therapy of a pathological condition or a pathological disorder comprising the steps of (a) contacting a sample comprising polyadenylated RNA with said drug candidate; (b) employing the steps of the method for the amplification of mRNA of the present invention on said sample; and (c) detecting the presence, the absence, the increase or the decrease of particular expressed genes in said sample.
The sample to be contacted with said drug candidate may be an isolated organ, tissue, (low number of) cells or a single cell. Said sample may also be a tissue or a cell culture sample. Furthermore, it is also envisaged that a laboratory animal and/or a subject may be contacted with said drug candidate and that after (or during) said contact a corresponding sample is obtained, for example, by biopsy.

Furthermore, the present invention provides for a method for in vitro detection of a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of (a) providing a sample comprising polyadenylated RNA from said subject; (b) employing the method for the amplification of mRNA of the present invention on said sample; and (c) detecting a pathological condition or a susceptibility to a pathological condition based on the presence, the absence, the increase, the decrease or the amount of (a) expressed gene(s) in said sample.
The presence, absence, increase or decrease or amount can be detected, inter alia, by comparing the obtained cDNA(s) with obtained cDNA(s) from a healthy control sample. The sample(s) may be of human origin.

In addition, the present invention relates to the use of the amplified cDNA as obtained by the method of the invention for in vitro and/or in vivo expression. Methods for in vitro and/or in vivo expression are well known in the art and are described, inter alia,

("Current Protocols in Molecular Biology", edited by Ausubel et al., John Wiley & Sons, USA (1988); Schoelke, Nature Biotech., 18, 233-234 (2000)) or in "Biotechnology"; edited by Rehn and Reed, VCM Verlagsgesellschaft mbH, Weinheim, FRG, (1993). Furthermore, in vitro expression in plant cells is described in Weissbach "Methods for Plant Molecular Biology", Academic Press, San Diego, U.S.A. (1988). Particular preferred systems for in vitro expression are translation systems known in the art, like E.coli lysates for coupled transcription/translation (Basset, J. Bacteriol.,(1983) 156, 1359-1362), wheat germ translations systems or reticulocyte lysates (Walter, Methods Enzymol., 93, 682-691 (1983); Dasnahapatra, Methods Enzymol., 217, 143-151 (1993); Hancock, Methods Enzymol, 255, 60-65 (1995); Wilson, Methods Enzymol., 250, 79-91 (1995)). Said in vitro and/or in vivo expression of said amplified cDNA comprises transcription as well as translation events and, therefore, comprises the generation of mRNA as well as, if desired, of protein(s) and/or peptide(s). Therefore, the present invention also relates to the use of amplified cDNA as obtained by the method of the present invention for the in vitro and/or in vivo preparation of mRNA transcripts.

The present invention also relates to the use of the amplified cDNA as obtained by the method of the present invention or of mRNA transcripts as defined hereinabove and obtained by in vitro and/or in vivo expression of the cDNA as obtained by the method of the present invention, in hybridization assays, and/or in interaction assays.

Preferably, said hybridizing assays are carried out under defined conditions. Most preferably, said hybridizing conditions are stringent conditions. However, the term "hybridizing" as used in accordance with the present invention relates to stringent or non-stringent hybridization conditions. Said hybridization conditions may be established according to conventional protocols described, for example, in Sambrook, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory (1989) N.Y., Ausubel, "Current Protocols in Molecular Biology", Green Publishing Associates and Wiley Interscience, N.Y. (1989), or Higgins and Hames (eds) "Nucleic acid hybridization, a practical approach" IRL Press Oxford, Washington DC, (1985).

In a preferred embodiment, said hybridization assay comprises the hybridization to oligonucleotide arrays, cDNA arrays, and/or PNA arrays, said interaction assay comprises the interaction with carbohydrate(s), lectin(s), ribozyme(s), protein(s), peptide(s), antibody(ies) or (a) fragment(s) thereof, and/or aptamer(s).

The above mentioned arrays are well known in the art (see, inter alia, Debouck, Nat. Genet. 21:48-50 (1999); Hacia, Nat. Genet., 21, 42-7 (1999); Cole, Nat. Genet. 21, 38-41 (1999); Bowtell DD., Nat. Genet., 21, 25-32 (1999); Cheung, Nat. Genet., 21, 15-19 (1999); Duggan, Nat. Genet., 21, 10-14 (1999); Southern, Nat. Genet., 21, 5-9 (1999)). In particular, cDNA arrays may be obtained from Clontech, Palo Alto; Research Genetics, Huntsville and comprise cDNA microarrays, and oligonucleotide arrays may be obtained from Affymetrix, Santa Clara. cDNA arrays may be prepared, inter alia, according to the methods described in DeRisi, Nat. Genet. (1996), 14, 457-460; Lashkari, Proc. Natl. Acad. Sci. USA, 94, 13057-13062 (1997); Winzeler, Methods Enzymol. 306, 3-18 (1999); or Schena (1995), loc. cit., oligonucleotide arrays, inter alia, according to Southern (1999), loc. cit.; Chee, Science, 274, 610-614 (1996). The above mentioned arrays may comprise macroarrays as well as microarrays.

As shown in the appended examples, the cDNA as obtained by the method of the present invention (or mRNA transcripts of said cDNA) can be employed on cDNA arrays/cDNA microarrays in order to deduce the gene expression pattern/transcriptome of a (test) sample comprising polyadenylated RNA.

Hybridization assays as described herein above are useful, inter alia, in medical, diagnostic, pharmacological as well as in scientific settings. As shown in the appended examples, it is possible to employ DNA as obtained by the method of the present invention in order to deduce the (gene) expression pattern of pathologically modified cells and/or tissues, e.g., tumerous (cells) tissues, restenotic tissue.

The appended examples document, inter alia, that the method of the present invention can be employed to deduce differentially expressed genes in restenotic tissue. In this way 224 genes were identified that are differentially expressed in restenosis, wherein 167 genes were overexpressed and 56 genes were underexpressed in comparison to controls. The detection of specific, differentially expressed genes or gene expression

pattern(s) can, therefore, also be employed in diagnostic methods in order to define, inter alia, restenotic tissue. Furthermore, as described in the appended examples, the method of the present invention may be useful in the diagnosis of neoplastic diseases, cancer.

The amplified cDNA as obtained by the method of the present invention is, therefore, particularly useful in establishing gene expression profiles of tissues and/or cells. Such gene expression profiles/gene expression patterns may be particularly useful and important in drug discovery screens. It is particularly preferred that data obtained by such gene expression profiling be used in combination with drug activity patterns (see, inter alia, Weinstein, Science 275 (1997), 343-349; Weinstein, Science 258 (1992), 447-451, van Osdol, J. Natl. Cancer Inst. 86 (1994), 1853-1859 or Pauli, J. Natl. Cancer Inst. 81 (1989), 1088-1092). Furthermore, it is envisaged that cDNA as obtained by the method of the present invention and/or mRNA transcripts thereof be used in assays wherein gene expression patterns and drug activity profiles are correlated as described in Scherf, Nat. Genetics 24 (2000), 236-244 and in Ross, Nat. Genetics 24 (2000), 227-235. Further, the "transcriptome"-data obtained by the methods of the invention, as described herein above, may also be correlated on the protein level, as demonstrated in the appended examples.

The present invention also relates to the use of amplified cDNA obtained by the method of the invention for sequence specific PCR, cDNA cloning, substractive hybridization cloning, and/or expression cloning. Specific PCR can be used, e.g., to determine the relative amounts of transcripts within a given sample and between samples. The cDNA generated by the present invention could also be applied to subtractive hybridization cloning to select for cDNAs specific for or absent from the sample which is demonstrated in the appended examples (Rothstein, Methods Enzymol. 225, 587-610 (1993); Diatchenko, Methods Enzymol. , 303, 349-380 (1999)).
In a preferred embodiment, the adapter-primers Eco 44 I : 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CTC GCC CGG GCA GG-3' (SEQ ID NO: 31), Eco 12 I : 5'-AAT TCC TGC CCG-3' (SEQ ID NO: 32), Eco 43 II : 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT GGT GCG GAG GGC G-3' (SEQ ID NO: 33) or Eco 12 II : 5'-AAT

TCG CCC TCC-3' (SEQ ID NO: 34) may be employed with the above mentioned method i.e. substractive hybridization analysis. In a further preferred embodiment, the primers P1-30 : 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG-3' (SEQ ID NO: 35), P2-30 : 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT-3' (SEQ ID NO: 36), P1-33: 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CTC-3' (SEQ ID NO: 37), P2-33: 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT GGT-3' (SEQ ID NO: 38), PN1-30 : 5'-CGA CTC ACT ATA GGG CTC GAG CGG CTC GCC-3' (SEQ ID NO: 39) or PN2-30 : 5'-GTG AAG ACG ACA GAA AGG TCG CGT GGT GCG-3' (SEQ ID NO: 40) may be employed when amplifying the resulting cDNA populations which may be obtained by the above mentioned substractive hybridization analysis.

In a more preferred embodiment primers primers P1-30 : 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG-3' (SEQ ID NO: 35), P2-30 : 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT-3' (SEQ ID NO: 36) are employed for the aforementioned method as shown in the appended examples.

The present invention also provides for a kit comprising at least one primer as defined herein above.

Advantageously, the kit if the present invention further comprises, besides said primer /primers, optionally, solid supports (such as magnetic beads), enzymes, such as reverse transcriptase(s), RNA-ligase or terminal deoxynucleotidyltransferase as well as (a) reaction buffer(s) (like cDNA "wash buffer" or "tailing buffer") and/or storage solution(s). Furthermore, parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. The kit of the present invention may be advantageously used for carrying out the method(s) of the invention and could be, inter alia, employed in a variety of applications referred to above, e.g., in diagnostic kits or as research tools. Additionally, the kit of the invention may contain means for detection suitable for scientific and/or diagnostic purposes. The manufacture of the kits follows preferably standard procedures which are known to the person skilled in the art.

The Figures show:

Fig. 1. Parameters determining amplification success. a) Twenty HT29 colon carcinoma cells ((ATCC: HTB-38) lanes 1-20) were individually isolated and mRNA reverse

transcribed in the presence of different concentrations of random hexamer primers (lanes 1-5, 80 µM; lanes 6-10, 8µM; lanes 11-15, 0.8 µM; lanes 16-20, 0.08 µM). 1/10 of the cDNA was subsequently tested for the detection of the ki-ras transcript by gene-specific PCR. b) Influence of the homopolymer tail on sensitivity. A 350 bp TGF-α fragment was isolated, diluted and either dA or dG tailed. Serial dilutions were tested by PCR using poly-dT or poly-dC containing primers, respectively, and a primer within the TGF-α sequence. The informative dilutions are shown in duplicates. (lanes 1+2, negative control; lanes 3+4, 10^{-3} dilution; lanes 5+6, 10^{-5} dilution). c) FL4-N6 primed and revers transcribed mRNA was dG-tailed and amplified using the CP3 + FL4 primers (lanes 1-3) or CP2 + FL4 primers at different annealing temperatures (lane 1+4, 68°C, lane 2+5 65°C, lane 3+6, negative control). d) An identical amount of mRNA as in c) was reverse transcribed using the CFL5cN6 primer, and amplified with the CP2 primer. An equal amount of cDNA as in c) (lane 3+4) resulted in amplification of a wide range of cDNA fragments as did a 1:200 dilution (lane 1+2) at different annealing temperatures (lane 1+3, 68°C; lane 2+4, 65°C; lane 5, negative control).

Figure 2. Gene specific PCR for β-actin and various MAGE transcripts using unamplified pooled cDNA of A431 cells as positive control (+) and amplificates of single A431 cells (lane 2-4 and 6-8) that were divided into two halves (a+b) before global PCR. Two independent experiments were performed (lane 1-4 and 5-8) with lane 1 and lane 5 being the negative controls for the global PCR.

Figure 3. CGH profiles of two normal leukocytes (red) and two MCF-7 breast cancer cells (blue) of which the genomic DNA was isolated from the supernatant after mRNA isolation. The chromosomal ratios of the normal cells are within the dashed lines, giving the threshold for significance, whereas the profiles of the cancer cells are similar with regard to their chromosomal deletions and amplifications.

Figure 4. CGH profile of cell B derived from a breast cancer patient with very small primary tumor (stage T1a). Chromosomal deletions are marked with a red bar left of and chromosomal gains with a green bar right of the chromosome symbol.

Figure 5. Diagram illustrating the common and differentially expressed genes of cell B, C and L.

Figure 6. Hybridisation of cell L (left) and the matrix of positions and names of immobilized cDNAs. Genes were spotted in duplicates in diagonal direction, with the blue gene symbols oriented from upper left to lower right and the red gene symbols oriented from upper right to lower left.

Fig. 7: Immunohistochemical stains of neointima from human coronary artery in-stent restenosis for v. Giesson (left panel) and smooth muscle alpha-actin (right panel). The shown experiment is a representative of 3 independent specimen. Bars indicate 100 µm.

Fig. 8: PCR with gene-specific primer for ß-actin (lanes 1), EF-1α (lanes 2) and α-actin (lanes 3) as a control for successful PCR amplification of the first strand cDNA generated from microscopic tissue specimen. Shown is one representative from each study group (right panel: patient B; left panel: control donor b). The position of three size markers (M) is shown.

Fig. 9: cDNA array analysis. The same array is shown with three independent hybridization experiments comparing mRNA isolated from neointima (panel A) or from control vessel (panel B), and in the absence of a biological sample (panel C). The cDNA array contained 588 genes including nine housekeeping genes and three negative controls [M13 mp 18 (+) strand DNA; lambda DNA; pUC18 DNA]. The experiment shown here is a representative of hybridization experiments with 10 neointima and 10 control specimen. Circles indicate four hybridization signals (A-D) differentially expressed between restenosis and control.

Fig. 10: Transcription profiles of microscopic samples from human in-stent neointima and control vessels. Each column represents a gene expression analysis of a single

specimen for 53 selected genes. An arrow indicated genes that show significant up- or downregulation in neointima versus control. Eight highly expressed housekeeping genes are shown on the bottom. One grey value corresponds to a signal intensity as shown at the bottom of the figure.

Fig. 11: Verification of differentially expressed mRNAs from cDNA arrays by gene-specific PCR. The size of the expected PCR fragment is indicated on the right.

Fig. 12: Immunhistochemical staining of neointima from carotid artery restenosis for the FKBP12 protein. The experiment shown is a representative of three independent experiments. The bars represents a distance of 100µm. Panel A shows a hematoxylin eosin staining, panel B-D shows staining for FKBP12 of the border zone between healthy media and neointima (panel B), of healthy control media (panel C) and neointimal tissue (panel D).

Fig. 13: cDNA array analysis of gene expression. Four Clontech Atlas microarrays, containing a total of 2435 human cDNAs, were hybridized with cDNA labeled with Dig-dUPT prepared from RNA from in-stent neointima (n=10) and from control media/intima (n=11) as described in Materials and Methods. Spots indicate the mean of the relative expression of the two examined groups. Panel A shows the expression of all examined genes in this study. Panel B shows expression of the 224 differentially expressed genes, that were more than 2.5-fold induced or reduced in neointima and showed a statistical significance $p<0.03$ in the Wilcoxon test. For this presentation, zero value were replaced by a value of 0.0001, as a zero value is not representable in a logarithmic scale.

Fig. 14: Cluster image showing the different classes of gene expression profiles of the two hundred twenty four genes whose mRNA levels were different between neointima and control. This subset of genes was clustered into four groups on the basis of their expression in different cell types. The expression pattern of each gene in this set is displayed here as a horizontal strip. Each column represents the average mRNA expression level of the examined group. For each gene, the average of the mRNA level

of neointima (n=10), of control (n=11), of proliferating CASMCs (n=2) and of blood samples (n=10) normalized to the mRNA expression level of the housekeeping genes is represented by a color, according to the color scale at the bottom. Group I contained genes only expressed in neointima specimen (Fig. 14A). Group II contained genes expressed simultaneously in neointima and proliferating CASMCs (Fig. 14B). Group III consisted of genes, whose mRNA were expressed in neointima as well as in blood (Fig. 14C). Group IV contained genes, whose mRNA was overexpressed in control specimen (Fig. 14D).

Fig. 15: Expanded view of the transcription factorcluster containing 14 genes that were upregulated in neointima versus control and three transcription factors that were downregulated in neointima. In this case, each column represents a single specimen, and each row represents a single gene

Fig. 16: Expanded view of the IFN-γ-associated cluster containing 32 genes that were upregulated in neointima versus control. In this case, each column represents a single specimen, and each row represents a single gene.

Fig. 17: Immunohistochemical stains of neointima from a carotid restenosis and healthy control media for the IRF-1 protein (left panel: control media; right panel: neointima). The experiment shown is a representative of 6 independent experiments.

Fig. 18: Immunohistochemical stain of neointima from a coronary in-stent for the IRF-1 protein. Panel A shows a hematoxylin eosin staining of the neointimal specimen from in-stent restenosis, panel B shows a staining for the smooth muscle cell marker α-actin, panel C shows the immunohistochemical stain for the transcription factor IRF-1 in neointima from in-stent restenosis and panel D shows immunohistochemical stain for CD3. The experiment shown here is a representative of three independent experiments.

Fig. 19: View of the IFN-γ-associated cluster containing the 32 genes that were upregulated in neointima versus control compared to expression in cultured CASMCs

and to cultured CASMCs stimulated for 16h with 1000 U/mL IFN-γ. In this case, each column represents a single specimen, and each row represents a single gene. One grey value corresponds to a signal intensity as shown at the bottom of the figure.

Fig. 20: Double staining of disseminated tumor cells in bone marrow. Cells in small aggregates (of seven and of two cells) in the upper panel and one single cell detected in bone marrow of two different patients were stained for cytokeratin (red fluorescence) and Emmprin (blue).

Fig. 21: Differential expression of the transferrin receptor (CD71) on tumor cells. DAPI staining of cellular nuclei (left panel), upregulated CD71 expression is found in tumor tissue (right panel).

Fig. 22: Effect of IFNγ on survival of cultured SMCs. Flow cytometry analysis of spontaneous (panel A and C) and H_2O_2-induced apoptosis (panel B and D). Cells were double-stained by FITC-labelled Annexin V and PI at 6 h after treatment with 100 µmol/l H_2O_2. A representative analysis of 5 independent experiments is shown.

Fig. 23: The effect of an IFNγ receptor null mutation on the development of neointima in a mouse model of restenosis. (A-D) Representative microphotographs of cross-sectioned mouse carotid arteries from wildtype (wt) and IFN-γR$^{-/-}$ knockout (ko) mice are shown for the untreated artery (control) and the contralateral ligated artery (ligated) at 4 weeks after ligation. The van-Giesson staining procedure was used. The bars represent a lenght of 100 µm. (E) Data from 16 wildtype and 11 IFN-γR$^{-/-}$ mice are shown as mean ± SEM (bars) and analyzed by the t-test for unpaired samples. The scale gives the thickness of media and neointima in µm. Open columns: control animals before and after carotis ligation; filled columns: knockout animals before and after carotis ligation. The shaded area indicates the thickness of neointima.

Fig. 24: Flow chart of SSH analysis performed with single cells or small cell samples.

Fig. 25: Screening of colonies by southern blot using labeled driver and tester as probes. Lane 1-9 colonies obtained after subtraction. Colony #4 was identified as ESE1, an epithelium-specific transcription factor. M = molecular weight marker.

Fig. 26: Differential expression of ESE1 in tumor cells analyzed by PCR and gelectrophoresis. Lane 1-4 single breast cancer cells, 5-7 bone marrow of healthy donors. M = molecular weight marker.

The invention will now be described by reference to the following biological examples which are merely illustrative and are not to be construed as a limitation of scope of the present invention.

Example I: Generation and global amplification of single cell cDNA

The amount of mRNA from single cells is too low for direct use in array-based transcriptome analysis. Total RNA from 50,000 cells (10µg) was reported to be the detection limit for direct-labelling approaches (Mahadevappa, Nat.Biotechnol., 17, 1134-1136 (1999)). Using a linear amplification step, this number could be reduced to 1000 cells (Luo, Nat. Med., 5,. 117-122 (1999)), which is still far beyond applicability for the study of micrometastatic cells. Thus reverse transcription of mRNA and amplification of the cDNA is necessary. Key is the development of an unbiased global amplification procedure. In a simplified manner, this approach consists of four basic steps: (1) isolation of the mRNA on oligo-dT-coated solid support, (2) cDNA synthesis using random primers containing a 5'-oligo-dC (or dG) flanking region, (3) 3'tailing reaction with dGTP (or dCTP) generating a 3'-oligo-dG flanking region, followed by (4) single primer-based amplification using a primer hybridizing to oligo-dG (or –dC) flanking regions of the cDNA molecules. In order to fulfil these four basic steps and to obtain high sensitivity and reliability for cDNA synthesis, 3'-tailing and pCR amplification, tRNA and rRNA had to be removed.

Furthermore, concentrations of random primers were 2000-8000-times higher for cDNA synthesis compared to previously desribed oligo-dT-based approaches (Brady, Methods. Enzymol., 225, 611-623 (1993); Trumper, Blood , 81, 3097-3115 (1993)), who employed 10 nM cDNA synthesis primers. Twenty HT29 colon carcinoma cells (ATCC: HTB-38) were individually isolated and processed. After cell lysis in cDNA synthesis buffer containing the detergent Igepal, groups of five cells were formed and reverse transcribed with four different concentrations of random cDNA synthesis primers. By gene-specific RT-PCR cDNA synthesis was tested for each concentration. Fig. 1a shows that higher concentrations of random primers for cDNA synthesis lead to increased detection rates of specific transcripts (e.g. ki-ras). Surplus primer, being an effective competitor of the subsequent tailing and amplification reaction, was, therefore, preferably removed prior to both steps. Equally, high dNTP concentrations improved cDNA synthesis but interfered with the subsequent tailing reaction and needed to be removed. Standard cacodylate-containing tailing buffer interfered with the following PCR and was replaced with a KH2PO4 buffer of low ionic strength (Nelson, Methods Enzymol., 68, 41-50 (1979). Capturing of mRNA on oligo-dT coated magnetic beads provided for simple handling during mRNA isolation and buffer exchange steps. In the following, the isolation of single cells, mRNA isolation, cDNA synthesis and 3'-tailing is briefly described and exemplified.

Tumor cells were isolated from bone marrow as described (Klein, Proc. Natl. Acad. Sci. USA, 96, 4494-4499 (1999)) . Briefly, viable bone marrow samples were stained for 10 min. with 10 µg/ml monoclonal antibody 3B10-C9 in the presence of 5% AB-serum to prevent unspecific binding. 3B10-positive cells were detected with B-phycoerythrin-conjugated goat antibody to mouse IgG (The Jackson Laboratory) and transferred to PCR-tubes on ice. Oligo-dT beads were added, the cells lysed in 10µl lysis buffer (Dynal), tubes rotated for 30 min. to capture mRNA. 10 µl cDNA wash buffer-1 (Dynal) containing 0.5% Igepal (Sigma) was added and mRNA bound to the beads washed in cDNA wash buffer-2 (50 mM Tris-HCl, pH 8,3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, supplemented with 0.5% Tween-20 (Sigma)), transferred to a fresh tube and washed again in cDNA wash buffer-1 to remove any traces of LiDS and genomic DNA. mRNA was reverse transcribed with Superscript II Reverse Transcriptase (Gibco BRL) using the buffers supplied by the manufacturer supplemented with 500 µM dNTP, 0.25%

Igepal, 30 µM Cfl5c8 primer (5'-$(CCC)_5$ GTC TAG ANN $(N)_6$-3') and 15 µM CFL5cT (5'-$(CCC)_5$ GTC TAG ATT $(TTT)_4$ TVN, at 44°C for 45 min. Samples were rotated during the reaction to avoid sedimentation of the beads. cDNA remained linked to the paramagnetic beads via the mRNA and washed once in the tailing wash buffer (50 mM KH_2PO_4,, pH 7.0, 1mM DTT, 0.25% Igepal). Beads were resuspended in tailing buffer (10 mM KH_2PO_4, pH 7.0, 4 mM $MgCl_2$, 0.1 mM DTT, 200 µM GTP) and cDNA-mRNA hybrids were denatured at 94 °C for 4 min, chilled on ice, 10 U TdT (MBI-Fermentas) added and incubated at 37°C for 60 min or 37°C, 60 min and 22°C over night. After inactivation of the tailing enzyme (70°C, 5 min), PCR-Mix I was added consisting of 4 µl of buffer 1 (Roche, Taq long template), 3% deionized formamide (Sigma) in a volume of 35 µl. The probes were heated at 78°C in the PCR cycler (Perkin Elmer 2400), PCR Mix II, containing dNTPs at a final concentration of 350 µM, CP2 primer (5'-TCA-GAA-TTC-ATG-CCC-CCC-CCC-CCC-CCC-3', final concentration 1.2 µM) and 5 Units of the DNA Poly-Mix was added, (Roche, Taq Long Template) in a volume of 5 µl for a hot start procedure. Forty cycles were run at 94°C, 15 sec, 65°C, 30°C, 68°C, 2 min for the first 20 cycles and a 10 sec- elongation of the extension time each cycle for the remaining 20 cycles, and a final extension step at 68°C, 7 min. These PCR amplification conditions differ substantially from Brail, Mut. Res. Genomics, 406, 45-54 (1999). Annealing temperature in Brail is only 42°C for 2 min in contrast to the 65°C applied in this example of method of invention.

Tailing efficiency as well as the sensitivity of the subsequent PCR of poly-dA- and poly-dG-tailed sequences was assessed using a defined cDNA fragment with a homopolymer tail of either poly-dA or poly-dT. The poly-(dA) and poly-(dG)-tailed fragments were diluted and then amplified by PCR using equal amounts of poly(dT) and poly(dC) primers, respectively. In these experiments poly-C primers binding to poly-G tails were found to be at least 100-times more sensitivity than poly-T primers on poly-dA tails (Fig. 1b compare lanes 1,2 to 3,4)

Various cDNA synthesis primers sharing the same poly-dC flanking region in combination with random hexamers (N6), octamers (N8), oligo-dT $(dT)_{15}$ alone or in combination were compared. All worked well and reliably. The best results were

obtained with a combination of poly-dC -N8 and poly-dC-$(dT)_{15}$ primers (data not shown).

The most dramatic improvement was obtained when only one primer (Fig. 1c) was used for global PCR instead of two (Fig. 1d). The cDNA synthesis primer consisted of a 3' random hexamer and flanking region either a poly-dC stretch (CFI5c) or a flanking sequence of all four bases (FI4N6).Two poly-dC binding primers were tested in combination with an additional primer binding to FI4 complementary sequence (Fig 1c). Use of an additional primer (FL4) to the poly-dC binding primers (CP2, CP3) prevented amplification (Fig. 1c, lanes 1,2 and 4,5). This is likely due to the high primer concentrations required for optimal sensitivity. The use of the CP2 primer alone resulted in amplification of a wide range of cDNA molecules (0.2-3 kB). Even highly diluted cDNA (1:200) was still sufficient for global amplification (Fig. 1d).

Example II: Transcriptome analysis of single cells: specificity, reproducibility, sensitivity, and suitability for cDNA array analysis

Isolated single cells from cultured cell lines were analyzed by the optimized protocol for cDNA synthesis, tailing and amplification. A total of 100 single cells have so far been successfully tested for ß-actin and EF-1α expression by gene-specific PCR (data not shown). cDNAs for housekeeping genes were found in a sufficient copy number per cell to be relatively independent of the region used for specific amplification in the secondary PCR. For less abundant transcripts, it was noted that the size of the chosen coding sequence determined detection rates. Highest sensitivity was obtained with the two primers being separated by less than 200 bp (data not shown).

The PCR amplificates from single cells were tested for suitability of cDNA array analysis. For this purpose, the obtained cDNA was Dig(Digoxigenin)-labeled. Dig-UTP was incorporated by PCR. For expression profiling 0.1-1µl of the original PCR amplified cDNA fragments were used for reamplification in the presence of digoxigenin-labeled dUTP (Boehringer Mannheim), 50 µM dig-dUTP, 300 µM dTTP, and other dNTPs at a final concentration of 350 µM. Reamplification conditions were essentially as described above, modifications were the use of 2.5 Units of the DNA Poly Mix. Initial denaturation at 94°C for 2 min. followed by 12 cycles at 94°C, 15 sec, 68°C, 3 min and a final extension time of 7 min. Specific transcripts were detected using 1 µl of a 1/10 dilution of the original PCR to a final volume of 10 µl.

The specificity of the hybridization of digoxigenin-labeled probes is depicted in Table 1, where the expression pattern of genes from single cells of different histogenetic origin are shown. Cells were MCF-7 (ATCC Number HTB-22), A431 (ATCC Number CRL-1555), K-562 (ATCC Number CCL-243), JY (International Histocompatibility Workshop: IHW9287). Only the MCF-7 and A431 cell expressed the cytokeratin genes, markers for their epithelial origin, whereas the erythroleukemia K562 cell and EBV-transformed B cell JY expressed genes of a haematopoetic origin, including CD33, CD37, CD38, and kappa light chain in the B cell. In addition, the testis- and tumor-specific MAGE genes were highly expressed in all cancer cells but not the virally transformed B cell. These data show that single cell PCR amplificates are useful for cDNA array analysis and produce cell type-specific gene expression patterns of single cells.

Table 1: Expression of histogenetically informative genes by single cells derived from different tissues.

Table 1

	MCF-7	A431	K562	JY
Aktin	+	+	+	+
EF-1a	+	+	+	+
CK7	+	+	-	-
CK10	-	+	-	-
CK13	-	+	-	-
CK18	+	+	-	-
CK19	+	+	-	-
EGP	+	+	-	-
CD33	-	-	+	+
CD37	-	-	+	+
CD38	-	-	+	-
Kappa	-	-	-	+
Vimentin	-	+	+	-
α-6 Integrin	+	-	-	-
ß-1 Integrin	+	-	-	-
ß-2 Integrin	-	-	-	+
ß-4 Integrin	-	-	+	-
ß-7 Integrin	-	-	-	+
FAK	+	-	-	-
Mage1	+	-	+	-
Mage2	+	+	+	-

Mage3	+	-	+	-
Mage6	+	-	+	-
Mage12	+	+	+	-

Individual cells grown in culture were isolated, cDNA synthesized, amplified and hybridized to an array of histogenetically informative genes. Cells were from the following cell lines MCF-7 (breast cancer); A431 (epidermoid carcinoma); K562 (chronic myeoloid leukemia); JY (Epstein-Barr virus transformed B cell line).

In order to assess reproducibility, the expression pattern of four MCF-7 cells were compared using a cDNA array Generation 4 with 110 different genes (Table 2). Custom made cDNA arrays were prepared as follows. cDNAs were PCR-amplified with gene-specific primers from human cDNA, PCR amplificates were gel-purified and 15 ng DNA per amplificate was spotted onto nylon membranes (Boehringer) using a BioGrid spotting robotic device (Biorobotics). DNA Macroarrays were termed Generation 4 and Generation 5 (see herein below).

Filter Generation 4: Spotted genes were:

Protein Name	HUGO Name	Protein Name	HUGO Name
Cytokeratin 7	KRT7	slap	SLA
Cytokeratin 8	KRT8	p21	CDKN1A
Cytokeratin10	KRT10	p68	
Cytokeratin13	KRT13	p27	CDKN1B
Cytokeratin18	KRT18	Eck	EPHA2
Cytokeratin19	KRT19	P33	ING1
Cytokeratin20	KRT20	B61	EFNA1
Emmprin II	BSG	p53 III	TP53
MT1-MMP	MT1-MMP	E-Cad	CDH1
MT2-MMP	MT2-MMP	p53 IV	TP53
MT3-MMP	MT3-MMP	P-Cad	CDH3
MT4-MMP	MT4-MMP	p57	CDKN1C

TIMP1	TIMP1	N-Cad	CDH2
TIMP2	TIMP2	Cyclin D	CCND1
TIMP4	TIMP4	c-myc I	MYC
MMP1	MMP1	Gas1	GAS1
uPA	PLAU	c-myc II	MYC
uPA-Rezeptor	PLAUR	KI-67	MKI67
PAI1	PAI1	RB	RB1
PAI2	PAI2	b-Aktin	ACTB
CathepsinB	CTSB	HTK	TK1
CathepsinD	CTSD	EF-1a	EEF1A1
CathepsinL	CTSL	RAD 51	RAD51
Stromelysin1	MMP3	A20	TNFAIP3
Stromelysin3	MMP11	Nck	NCK1
Gelatinase A	MMP2	BCL-2	BCL2
Gelatinase B	MMP9	pBS	
Matrilysin	MMP7	GAPDH	GHPDH
Cystatin1	CSTA	hEST	TERT
Cystatin 2	CSTB	Mage 1	MAGEA1
Cystatin 3	CST3	TSP-1	THBS1
ADAM 8	ADAM8	Mage 3	MAGEA3
ADAM 9	ADAM9	mrp-1	ABCC1
ADAM 10	ADAM10	Mage 4	MAGEA4
ADAM 11	ADAM11	mdr-1	ABCB1
ADAM 15	ADAM15	Mage 6	MAGEA6
ADAM 20	ADAM20	DEP-1	PTPRJ
ADAM 21	ADAM21	Mage 12	MAGEA12
TACE	ADAM17	PTP-μ	PTPRM
a4-Integrin	ITGA4	Mage1F	MAGEA1
a5-Integrin	ITGA5	Creatin Kinase	CKM
a6-Integrin	ITGA6	Mage2F	MAGEA2
av-Integrin	ITGAV	Mage 4F	MAGEA4
GFP		Mage3F	MAGEA3
beta-Actin	ACTB	Mage 12F	MAGEA12
b1-Integrin	ITGB1	CD16	FCGR3A
b2-Integrin	ITGB2	TGF-a	TGFA
b3-Integrin	ITGB3	CD33	CD33

b4-Integrin	ITGB4	TGF-b	TGFB1
b5-Integrin	ITGB5	CD34	CD34
b7-Integrin	ITGB7	VEGF	VEGF
p15	CDKN2B	CD37	CD37
Fak	PTK2	IGF-I	IGF1
p16	CDKN2A	CD38	CD38
Ramp-1		kappa	IGKC
CD40	CD40	TGF-b R.II	TGFBR1
Ramp-2		lambda	IGLC1
CD45 II	PTPRL	IGF-RI	IGFR1
EMM I	BSG	Vimentin	VIM
CD83	CD83	IGF-RII	IGFR2
GFP		EGP-1	M4S1
pBS		MUC 18	MCAM
erb B2	ERBB2	DP-I	DSP
TCR	TCRA	PHRIP	PHLDA1
TGF-b Rez.I	TGFBR1	CEA	CEA
		EF-1a	EEF1A1

Table 2: Commonly and differentially expressed genes of four single MCF-7 cells.

Table 2

4/4	3/4	2/4	1/4
EF-1a	CK19	Beta-4-Integrin	CK10
GAPDH	TIMP-1	Beta-5-Integrin	CK13
b-Actin	Cathepsin B	P53	ADAM 9
CK7	Cathepsin D	Creatin Kinase	ADAM 15
CK8	Cathepsin L		ADAM17 (TACE)
CK18	ADAM 10		p16
CK20	c-myc		p21
Alpha 6-Integrin			p27
Beta1-Integrin			p33
Fak			ki-67

EMMPRIN
u-PAR
Matrilysin
Cyclin D1
Eck
EpCAM
Mrp-1
PHRIP
hTK
E- cadherin
IGF-R I
IGF-R II
TGF-beta
VEGF
DP-I

Heterogeneity of gene expression of individual cells derived from the same cell clone. Four MCF-7 cells isolated from cell culture were analyzed by single cell analysis of gene expression. Listed are the transcripts that were detected in all four single cells (4/4), three of four (3/4), two of four (2/4), and one of four (1/4). 18/46 (39%) expressed genes were detected in all cells. 61% genes could only found in a portion of the four cells. 63 genes were negative for all cells tested.

46 genes (42%) were expressed in at least one cell and 63 (58%) were negative for all four cells. Eighteen of the 46 (39%) expressed genes were detected in all four cells whereas the remaining 29 (61%) were found to be heterogeneously expressed. To evaluate whether this heterogeneity was due to intercellular variation or is an artifact of the technique, it was tested whether disparity is also observed with the cDNA of a single A431 cell that was split for two separate PCR amplifications. In a first experiment, gene-specific PCRs with the globally amplified PCR products obtained from 50% of single cell cDNA (Fig. 2) were performed. For comparison, cDNA isolated from a pool of 500.000 A431 cells were diluted to such an extent that the intensity of the ß-actin band was similar to that obtained with 50% of the single cell cDNA. After 32 cycles and with a cDNA amount corresponding to about 10.000 cells, the ß-actin signal of the pool control and 50% of the single cell cDNA reached the plateau phase of amplification. As shown in Figure 2, the variation between two cDNA halves of the same cell was very low. In two independent experiments, each half (a+b) from six A431 cells yielded ß-actin bands of similar intensity.

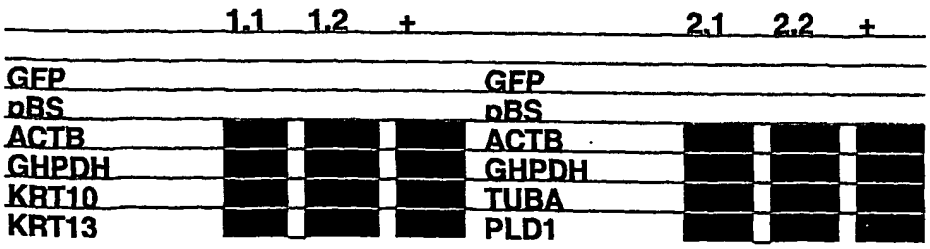
In order to test the reliability of the global amplification of the cDNA, a second gene sequence-specific PCR amplification was perfomed. As the efficiency of gene-specific PCR amplification is known to be primer sequence-dependent, the amplification of MAGE transcripts was tested, which are very demanding with respect to primer design (Kufer, WO98/46788 (1998); Serrano, Int. J. Cancer 83, 664-669 (1999)). The level of MAGE expression determined by sequence specific PCR was consistently lower than that of beta-actin. The relative abundance of MAGE transcripts in split single cell samples after global PCR amplification of the cDNA (Fig. 2, lanes 2-4 and 6-8) was comparable to that of the control sample from unamplified cDNA from pooled cells (Fig. 2, +). In 4 out of 6 cases, the results were identical for both halves of the cDNA. The lack of any MAGE transcript in cell half 7a and 8b most likely indicates an unequal distribution of the cDNAs between the two halves.

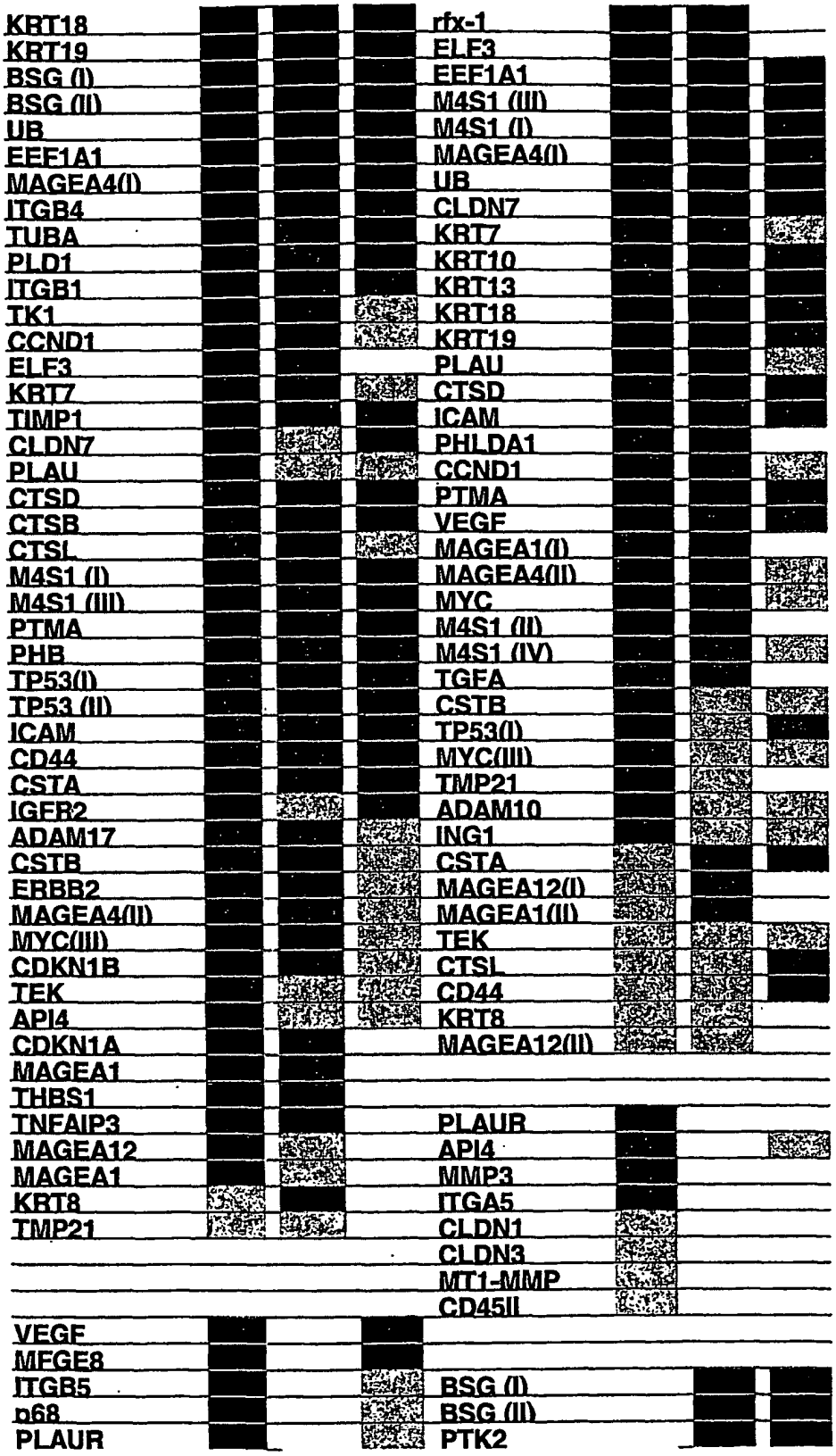
The observed sequence-independent amplification is characteristic of the poly-dC primer, which contains fifteen cytosine residues and therefore introduces primer binding sites with equally high CG- content. The experimental conditions suited for such a primer, i.e. high annealing temperature (65°C) in the presence of 3% denaturing formamide, lead, to a remarkable reproducibility and did not introduce major quantitative changes to the single cell transcriptome.

Amplificates from split single cell cDNAs and, as control, cDNA from 5,000 pooled cells were labeled and hybridized to a cDNA array representing 193 different genes. Most transcripts could be detected with both halves of the single cell amplificates (Tab. 3).

Table 3: Gene expression patterns of single cells split in two pools of cDNA prior to global PCR compared to pooled cDNA of 5000 cells.

Table 3




KRT18
KRT19
BSG (I)
BSG (II)
UB
EEF1A1
MAGEA4(I)
ITGB4
TUBA
PLD1
ITGB1
TK1
CCND1
ELF3
KRT7
TIMP1
CLDN7
PLAU
CTSD
CTSB
CTSL
M4S1 (I)
M4S1 (III)
PTMA
PHB
TP53(I)
TP53 (II)
ICAM
CD44
CSTA
IGFR2
ADAM17
CSTB
ERBB2
MAGEA4(II)
MYC(III)
CDKN1B
TEK
API4
CDKN1A
MAGEA1
THBS1
TNFAIP3
MAGEA12
MAGEA1
KRT8
TMP21
VEGF
MFGE8
ITGB5
p68
PLAUR
rfx-1
ELF3
EEF1A1
M4S1 (III)
M4S1 (I)
MAGEA4(I)
UB
CLDN7
KRT7
KRT10
KRT13
KRT18
KRT19
PLAU
CTSD
ICAM
PHLDA1
CCND1
PTMA
VEGF
MAGEA1(I)
MAGEA4(II)
MYC
M4S1 (II)
M4S1 (IV)
TGFA
CSTB
TP53(I)
MYC(III)
TMP21
ADAM10
ING1
CSTA
MAGEA12(I)
MAGEA1(II)
TEK
CTSL
CD44
KRT8
MAGEA12(II)
PLAUR
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MMP3
ITGA5
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CLDN3
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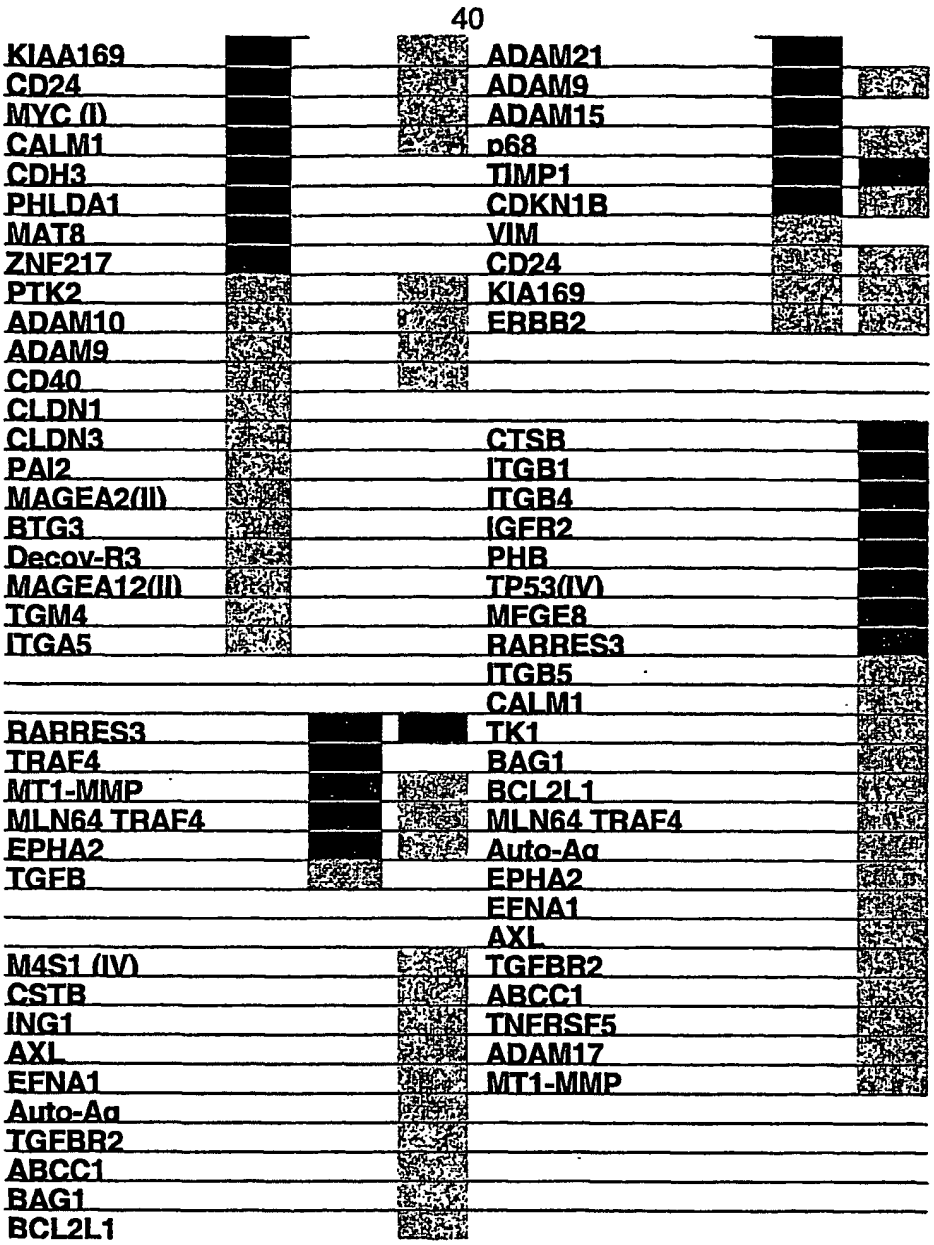
CD45II
BSG (I)
BSG (II)
PTK2



The cDNAs of two single cells were split prior to PCR amplification and compared to a cDNA pool derived from 5000 cells. All cDNAs were amplified by global PCR and analyzed by hybridization to a cDNA array. The gene expression profiles of the corresponding halves (1.1 and 1.2; 2.1 and 2.2) are juxtaposed to the cell pool (+). The genes are listed according signal strength (the darker, the stronger) and detection in both halves of the same cell. The filter used was Generation 5, genes and protein

names are listed below (for preparation of said Generation 5 filter, see herein above (Generation 4 filter)).

Generation 5 Filter:

Protein	HUGO
A20	TNFAIP3
a4-Int	ITGA4
a5-Int	ITGA5
a6-Int	ITGA6
ADAM10	ADAM10
ADAM15	ADAM15
ADAM21	ADAM21
ADAM9	ADAM9
Auto-Ag	SHGC-74292
av-Int	ITGAV
Axl	AXL
b1-Int	ITGB1
b2-Int	ITGB2
b3-Int	ITGB3
b4-Int	ITGB4
b5-Int	ITGB5
B61	EFNA1
b7-Int	ITG7
BA46	MFGE8
BAG1	BAG1
b-Aktin	ACTB
b-Casein	CSN2
Bcl-2	BCL2
Bcl-xl	BCL2L1
b-micro	MSMB
BTG-3/ANA	BTG3
Calmodulin	CALM1
Cathepsin B	CTSB
Cathepsin D	CTSD
Cathepsin L	CTSL
CD16	FCGR3A
CD24	CD24
CD33	CD33
CD34	CD34
CD37	CD37
CD38	CD38
CD40	TNFRSF5

Protein	HUGO
CD44	CD44
CD45	PTPRC
CD83	CD83
CEA	CEA
CK10	KRT10
CK13	KRT13
CK18	KRT18
CK19	KRT19
CK7	KRT7
CK8	KRT8
Claud1	CLDN1
Claud3	CLDN3
Claud7	CLDN7
c-myc	MYCBP
Cyclin D1	CCND1
Cystatin A	CSTA
Cystatin B	CSTB
Decoy-R2	TNFRSF10D
Decoy-R3	TNFRSF6B
DEP-1	PTPRJ
DP-1	DSP
E2F6	E2F6
E-Cad	CDH1
Eck	EPHA2
EF1a	EEF1A1
EGP1	M4S1
Emmprin	BSG
EPC-1	PEDF
erbB2	ERBB2
Ese1b/ELF3	ELF3
Fak	PTK2
FGFR1	FGFR1
FGFRII	FGFR2
Gadd45	GADD45A
GAPDH	GHPDH
Gas1	GAS1
Gas6	GAS6
GFP	
hEST	TERT
Hevin	HEVIN
HTK	TK1
ICAM	ICAM
IGF RI	IGFR1
IGF RII	IGFR2

Protein	HUGO
Kappa	IGKC
Ki67	MKI67
KIA169	
Lambda	IGLC1
lot1/hZAC	Hs.75825
Mage1	MAGEA1
mage12	MAGEA12
Mage2f	MAGEA2
Mage4	MAGEA4
MAT8	PLML
Mdr-1	ABCB1
MLN62	TRAF4
MLN64 TRAF4	
mrp-1	ABCC1
MT1-MMP	MT1-MMP
Muc 18	MCAM
N-Cad	CDH2
Nck	NCK1
p15	CDKN2B
p16	CDKN2A
p21	CDKN1A
p27	CDKN1B
p33	ING1
p53III	TP53 (III)
p53IV	TP53 (IV)
p57	CDKN1C
p68	
PAI-2	PAI2
pBS	
P-Cad	CDH3
Phospholipase	PLD1
Phrip	PHLDA1
PIP	PIP
Prohibitin	PHB
Prost.Spec.Homeo.	Hs.73189
Prost.Spec.Transglu	TGM4
Prost.Spec.Uro.	UPK3
Prothym alpha	PTMA
PSA	KLK3
PTHrP	
PTP-μ	PTPRM
RB	RB1

Protein	HUGO
rfx-1	RFX1
Slap	SLA
Stromelysin 1	MMP3
Survivin	API4
TACE	ADAM17
TCR	TCRA
TGF-alpha	TGFA
TGF-beta	TGFB1
TGFB-RI	TGFBR1
TGFB-RII	TGFBR2
TIE-2/Tek	TEK
TIG3	RARRES3
Timp1	TIMP1
TMP21	TMP21
TSP-1	THBS1
Tubulin-a	TUBA
Ubiquitin	UB
uPA	PLAU
uPA-R	PLAUR
VEGF	VEGF
Vimentin	VIM
VLDLR	VLDLR
ZNF217	ZNF217
	Hs.46452

A total of 148 signals were obtained for the four cDNA halves. Of these, 95 (64%) were found in the corresponding halves, whereas 53 (36%) were found in only one half. Out of the 53 single positive signals 46 (87%) represented very low-abundant transcripts, with 26 (49%) not detectable and 20 (37%) only weakly expressed in the control of pooled cells. Seven genes (AXL, BAG1, BCL2L1, SHGC-74292, B61, TGFBR2 and ABCC1) were exclusively detected in the pooled sample, though with a rather weak signal. In contrast, 33 genes were only found in the half-cell experiments but not in the control. The signal intensity of the both halves was quite similar, with 55% and 76% of the signals having the same strength in the corresponding halves. Signals that were not identical in two corresponding halves may arise from of a non-random distribution of cDNA fragments prior to PCR. Particularly transcripts present in low (<10) copy number may be subject to such a distribution effect which, however, may not be obtained if samples are not split.

Example III: Combined transcriptome and genome analysis from single cells

A method of CGH (comparative genomic hybridization) analysis of single cells (SCOMP) was recently described (Klein, Proc. Natl. Acad. Sci. USA, 96, 4494-4499 (1999)). Using this method, a tumor cell can unambiguously be identified by its chromosomal aberrations. It was therefore attempted to isolate both genomic DNA and mRNA from the same cell. Isolated single cells were lysed in 10µl lysis buffer (Dynal) and tubes rotated for 30 min. to capture mRNA. 10 µl cDNA wash buffer-1 (50 mM Tris-HCl, pH 8,3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, supplemented with 0.5% containing 0.5% Igepal (Sigma)) was added and mRNA bound to the beads washed in cDNA wash buffer-2 (50 mM Tris-HCl, pH 8,3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, supplemented with 0.5% Tween-20 (Sigma)), transferred to a fresh tube and washed again in cDNA wash buffer-1 to remove any traces of LiDS and genomic DNA. mRNA was reverse transcribed with Superscript II Reverse Transcriptase (Gibco BRL) using the buffers supplied by the manufacturer supplemented with 500 µM dNTP, 0.25% Igepal, 30 µM Cfl5c8 primer (5'-$(CCC)_5$ GTC TAG ANN $(N)_8$-3') and 15 µM CFL5cT (5'-$(CCC)_5$ GTC TAG ATT $(TTT)_4$ TVN, at 44°C for 45 min. Samples were rotated during the reaction to avoid sedimentation of the beads. Primers used and mentioned in Fig.1 c and d were Cfl5cN6 (5'-$(CCC)_5$ GTC TAG ANN $(N)_6$-3') and FL4N6 5'-TTT CTC CTT AAT GTC ACA GAT CTC GAG GAT TTC $(N)_6$-3'). cDNA remained linked to the paramagnetic beads via the mRNA and washed once in the tailing wash buffer (50 mM KH_2PO_4,, pH 7.0, 1mM DTT, 0.25% Igepal). Beads were resuspended in tailing buffer (10 mM KH_2PO_4, pH 7.0, 4 mM $MgCl_2$, 0.1 mM DTT, 200 µM GTP) and cDNA-mRNA hybrids were denatured at 94 °C for 4 min, chilled on ice, 10 U TdT (MBI-Fermentas) added and incubated at 37°C for 60 min or 37°C, 60 min and 22°C over night. After inactivation of the tailing enzyme (70°C, 5 min), PCR-Mix I was added consisting of 4 µl of buffer 1 (Roche, Taq long template), 3% deionized formamide (Sigma) in a volume of 35 µl. The probes were heated at 78°C in the PCR cycler (Perkin Elmer 2400), PCR Mix II, containing dNTPs at a final concentration of 350 µM, CP2 primer (5'-TCA-GAA-TTC-ATG-CCC-CCC-CCC-CCC-CCC-3', final concentration 1.2 µM) and 5 Units of the DNA Poly-Mix was added, (Roche, Taq Long Template) in a volume of 5 µl for a hot start

procedure. Forty cycles were run at 94°C, 15 sec, 65°C, 30°C, 68°C, 2 min for the first 20 cycles and a 10 sec- elongation of the extension time each cycle for the remaining 20 cycles, and a final extension step at 68°C, 7 min.. PCR primers used in Fig 1 c were CP3 (5'- GCT GAA GTG GCG AAT TCC GAT GCC $(C)_{12}$-3') and FL4 (5'-CTC CTT AAT GTC ACA GAT CTC GAG GAT TTC-3').

The supernatants from the cell lysis and all washing steps (cDNA wash buffer 1 and 2) of the mRNA isolation were collected (total volume 60 µl). After transfer to a silanised tube the genomic DNA was ethanol precipitated overnight at –20°C in the presence of 20µg glycogen (Roche). All subsequent steps were performed as published (Klein, (1999), loc. cit.).

A major concern was incomplete precipitation of genomic DNA eventually leading to losses of DNA as seen with chromosome deletions in cancerous cells. However, experiments with cells of a defined karyotype clearly showed that either the cellular DNA was totally lost (30% of cases) or completely precipitated (70%) (data not shown). The complete recovery of genomic DNA may be due to the fact that interphase chromosomes are extensively interwoven so that either all or none is precipitated. The loss of all DNA is probably introduced by the change of reaction tubes during the separation of genomic DNA and mRNA. The karyotypes of two normal and two MCF-7 breast cancer cells whose DNA had been precipitated are shown in Figure 3. The profiles of the two normal cells showed no significant deviation from the midline while the multiple genomic aberrations of the two MCF-F7 cells were almost identical. Hence, malignant EpCAM-positive cells can be unambiguously distinguished by their genomic phenotype from normal EpCAM-positive cells in the bone marrow. This is of particular importance since EpCAM- expression is insufficient proof for the (malignant) identity of tumor cell(s) in bone marrow samples. It has to be noted that healthy donors also showed 0.5-5‰ "3 3B1O-C9-positive cells (3B10-C9, Prof. Judy Johnson, Institute for Immunology, Munich) is a high affinity mAb against EpCAM) when determined by immunofluorescence.

Example IV: Activity-related gene expression In three micrometastatic cells

Single tumor cells were isolated from three patients with different tumors and disease stages. The first patient (C) had a 10-year history of cervical carcinoma and presented with a suspicious finding on chest x-ray. In the second patient (L), an adenocarcinoma of the lung had recently been diagnosed which was post-operatively staged as pT2, N3, M0. The bone marrow sample was obtained during the anesthesia prior to the operation. The third sample was aspirated from the pelvic crest of a 31-year old breast cancer patient (B) whose disease was in the stage pT1a, pN1a (1/18), M0. Because of a local relapse, the histological G3 grading, and finding of one cytokeratin-positive cell in the bone marrow, this patient received high-dose chemotherapy (HD). The bone marrow sample was taken one month after completion of HD. SCOMP was performed with all three cells and showed multiple chromosomal aberrations verifying the cancerous origin of cells (Tab. 4).

Table 4: Genomic aberrations of 3B10-C9-positive cells isolated from bone marrow of a three patients with cervical carcinoma (C), lung cancer (L) and breast cancer (B).

Table 4

Cell	1p	1q	2p	2q	3p	3q	4p	4q	5p	5q	6p	6q
C		G				G				G/L		
L	L	G			L			L	G	L	G	G
B				G				G				

Cell	7p	7q	8p	8q	9p	9q	10p	10q	11p	11q
C						L	G			
L	G	G	L	G	L		L		L	G
B					L	L			L	L

Cell	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q
C		L		L			L			G	
L			L		L	L	G	L	L		
B	G	G		G							

Cell	19p	19q	20p	20q	21p	21q	22p	22q	Xp	Xq	Y
C			L	L				L			
L						G			G	G	
B									G	G	

Summary of the CGH-data obtained from the three micrometastatic cells. Losses (L) and gains (G) on the small (p) and long (q) arm of each chromosome are given for each cell.

The cell from patient B, who had the least advanced disease, showed the lowest extent of chromosomal changes (Fig. 4).

mRNA was isolated from all three cells and samples generated for SCAGE as described above. As control, the procedure was performed without the addition of a cell. cDNA amplificates were hybridized to Clontech Cancer 1.2 filters and to newly generated arrays (Axxima A6, Martinsreid) comprising a total of 1,300 genes.

Non-radioactiv hybridization to nylon filters was carried out as follows:
15 ng of the different PCR-amplified and subcloned cDNA fragments were spotted on positively charged nylon filters by Axxima AG, Martinsried. Filters were pre-hybridized overnight in the presence of 50 µg/ml E. coli and 50 µg/ml pBS DNA in 6 ml Dig-easy Hyb buffer (Roche Biochemicals). 9 µg of labeled PCR products from single cells were mixed with 100 µg herring sperm, 300 µg E. coli genomic DNA and 300 µg, denatured for 5 min at 94 °C, added to 6 ml Dig-easy hybridization buffer and hybridized for 36 hours. Stringency washes were performed according to the Roche digoxigenin hybridization protocol adding two final stringency washes in 0.1x SSC +0.1 % SDS for 15 min at 68°C. Detection of filter bound probes was performed according to the Digoxigenin detection system protocol supplied with the kit (Roche).

Only three genes had to be excluded from analysis because a signal was obtained in at least one of the negative controls. These genes were the VHL-binding protein, caspase 10, TGF-ß and hemoglobin α. The number of positive signals ranged from 5.3% (70/1313), 7.0% (92/1313) to 11.8% (155/1313) for cells from patients B, C, and L respectively. These numbers were considerably lower than those from single *in vitro*-grown carcinoma cells where signals were obtained with 10-20% of genes (data not shown). All three tumor cells expressed genes known to play a role in regulation of proliferation, replication or growth arrest (Fig. 5; Tab. 5).

Table 5: Upregulated genes implied in cell cycle status in cells C, L and B.

Table 5

Role in cell cycle	C	B	L
Positive regulators	RFC3	RFC3	RFC3
	LIG1	LIG1	
	STK12	STK12	
	P2G4	P2G4	
	RFC2	RFC2	
	ADPRT	ADPRT	
	S100A4	S100A4	
	CCNA (cyclin A)	CDC25	
	MKI67 (Ki-67)	VRK2	
	CENPF	DYRK4	
	D123	PRIM1	
	PIN1	PRKDC (DNA-PK)	
	EB1	CHD3	
	CDC27HS		
	CALM1		
	UBL1		
	TOP2A		
	HMGIY		
	HDAC3		
	RBBP4		
Negative regulators		CDKN1A (P21)	CDKN1A (P21)
		ING1	ING1
		DDIT1 (GADD45)	CDKN2A (P16)

Cells C and B expressed several positive regulators of the cell cycle, while only B and L expressed cell cycle inhibitors.

Cell C expressed the highest number of genes important for cell cycle progression, including cyclin A (CCNA), EB1, RC2, P2G4, PIN1, RBBP4 and CENPF. As most of these genes are tightly transcriptionally regulated and their mRNAs are rapidly degraded as cell division progresses, their expression not only indicates that cell C was engaged in cycling but can be faithfully captured in this activity by SCAGE.

Cell B expressed a number of genes important for replication as well as cell cycle inhibition. The pattern of transcripts suggests that the cell was in a state of DNA repair. The coexpression of GADD45 (DDIT1) and p21 (CDKN1A) are indicative for growth arrest (Smith, Science, 266, 1376-1380 (1994)) . Likewise, the expression of positive cell cycle regulators such as DNA-PK, RFC2, LIG1, ADPRT and PRIM1 has been implicated in DNA repair (Lindahl, Science, 286, 1897-1905 (1999); Barnes, Cell, 69,

495-503 (1992), Mossi, Eur. J. Biochem., 254, 209-216 (1998); Lee, Mol. Cell Biol. 17, 1425-1433 (1997)). As this cell survived an alkalyting, genotoxic high dose chemotherapy its expression profile may be interpreted as if re-entry into cell cycle was obviated. This interpretation is supported by the expression of pro-apoptotic genes such as caspase-6 and BAD that were only found with this cell. Execution of apoptosis in this cell may however be counteracted by expression of survivin (API4) (Fig. 5; Tab. 5).

The transcriptome obtained from cell L showed traits compatible with its engagement in dissemination and EMT. While gene expression of cell L did not resemble that of a cycling or DNA-repairing cell (see above) its 84 differentially expressed genes are mostly involved in cytoskeletal reorganization, cell adhesion and extracellular proteolytic activity (Tab. 6; Fig. 6).

Table 6: Upregulated genes in cell L indicative for an Invasive phenotype.

Table 6

cytoskeletal organization	Adhesion	proteolytic activity
Cytokeratin 2	Integrin alpha 3	Cathepsin B
Cytokeratin 6	Integrin alpha v	Cathepsin D
Cytokeratin 7	Integrin beta 2	Cathepsin L
Cytokeratin 8	Integrin beta 3	MMP7
Cytokeratin 10	Integrin beta 7	MT1-MMP
Cytokeratin 13, 15, 17		MT2-MMP
Cytokeratin 18	Cytohesin 1	uPA
Cytokeratin 19	Focal adhesion kinase	uPA-R
Vimentin	Desmoglein 2	ADAM 8
Beta-actin	E-cadherin	ADAM 15
	CD9	ADAM 17
RhoA		Bikunin
RhoB		
Rho-GDI2		Cystatin 2
A-raf		EMMPRIN
RAP-1A		
Cdc42		
Rac1		
P160 ROCK		
Ste20-like kinase		
Beta-catenin		

The present study analyzed for the first time cellular activities of individual tumor cells derived from the bone marrow of cancer patients. Cell C was derived from a cervical carcinoma patient who presented with lung metastasis after a ten-year latent period. This cell was found in proliferation. Cell B was from bone marrow of a breast cancer patient with a rather small primary cancer who had received high dose chemotherapy because of the apparent aggressiveness of her tumor. This cell showed relatively few and discrete genomic changes, a finding that is of particular interest with regard to the genomic changes required for dissemination. Moreover, this cell must have survived four cycles of a regular chemotherapy consisting of Epirubicin and Taxol in addition to a high-dose chemotherapy regimen involving alkylating agents. The obtained expression profile is diagnostic for growth arrest and ongoing DNA repair.

Most informative with respect to the process of dissemination was the transcriptome of cell L. Detected in a bronchial cancer patient without clinically manifest metastasis, this cell expressed many genes encoding proteins involved in active migration and invasion. Most of the activation cascade of the uPA system was found expressed, consisting of the cathepsin B, D, L, the uPA receptor and uPA itself. Likewise, genes involved in organizing filopodia, lamellipodia and stress fibers, the Rho family members RhoA and B, Rac1, Cdc42 and p160 rock, and genes encoding several adhesion molecules were upregulated in this cell. Its cytoskeleton seemed to undergo remodeling as shown by expression of many cytokeratins and vimentin, a marker for EMT.

It is noteworthy that the number of transcripts in single cells isolated from cultured cell lines was considerably lower than that from patient-derived tumor cells. This difference may speak for a tighter *in vivo* control of transcription that may become more relaxed when cells are grown in cell culture, e.g., by increased DNA demethylation. Expression analysis of *ex-vivo* specimen might therefore be much more informative than studies on cell lines. The minimal number of cells that has been used for cDNA array analysis so far was in the range of 1,000 cells (Luo (1999), loc. cit.). The sensitivity of the array hybridization might be further increased by longer immobilized cDNA fragments (fragment length on Clontech arrays is about 200 bp), and the amount of information

obtained by using glass chips with higher density and complexity. Although the present study analyzed only 1,300 genes, one has to consider that expression of only nine proteins has thus far been reported for micrometastatic cells. These proteins are ErbB2, transferrin receptor, MHC class I, EpCAM, ICAM-1, plakoglobin, Ki-67, p120 and uPA-receptor/CD87 (Pantel, J. Natl. Canc. Inst. 91, 1113-1124 (1999)).

The here described method has potential for the study of gene expression by rare cells in many other fields (as shown hereinbelow; for example, in the investigation of human restenotic tissue). For instance, the investigation of spatially and temporally regulated gene expression in embryogenesis and the analysis of stem cells and differentiated cells in adult tissues could be performed. Single cell analysis would greatly advance the understanding of atypical proliferation, metaplasia, pre-neoplastic lesions and carcinomata in situ.

A synopsis of genomic aberrations and the expression profiles of the same cell may reveal the contingencies of different genotypes and phenotypes within a tumor cell population.

High-dose chemotherapy, surgery, and anti-angiogenic therapy approaches can target rapidly dividing cells and large tumor masses but are ineffective in the elimination of remnant cells leading to minimal residual disease. Adjuvant therapies, like antibody-based approaches Riethmuller, J. Clin. Oncol., 16, 1788-1794 (1998), are still based on protein targets identified on the primary tumor. The here shown approach provides now an opportunity to discover targets for minimal residual disease by analyzing the micrometastatic cells directly.

Example V: Aberrant Gene Expression in human restenotic tissue

The above described method was furthermore employed to detect differentially expressed genes in human restenotic tissue.

A high rate of restenosis is significantly limiting the success of percutaneous transluminal coronary angioplasty with subsequent stent implantation as a frequent treatment of coronary atherosclerotic disease. Although several cellular and molecular mechanisms have been identified in the development of in-stent restenosis, specific targets for an effective therapeutic prevention of restenosis are still scarce. In this study differentially expressed genes in microscopic atherectomy specimen from human in-stent restenosis were identified. Immunohistochemistry showed that the restenotic material consisted mainly of smooth muscle cells (SMC) with rare infiltrates of mononuclear cells. cDNA samples prepared from restenotic specimen (n=10) and, as control, from intima and media of healthy muscular arteries (n=10) were amplified using a novel polymerase chain reaction protocol and hybridized to cDNA arrays for the identification of differentially expressed genes. Expression of desmin and mammary-derived growth inhibitor was downregulated, whereas expression of FK506-binding protein 12 (FKBP12), thrombospondin-1, prostaglandin G/H synthase-1, and the 70-kDa heat shock protein B was found to be upregulated with high statistical significance in human neointima. Using immunohistochemistry, FKBP12, a negative regulator of TGF-ß signaling, was also upregulated at the protein level in neointima providing a rationale for the therapeutic effect of the FKBP12 ligand rapamycin in the treatment of a porcine restenosis model.

To gain further insight into transcriptional and signaling events governing smooth muscle cell migration, proliferation and synthesis of extracellular matrix, differential gene expression screening was employed using cDNA array technology with probes generated from microscopic specimen of human restenotic tissue. The power of this technology is the ability to simultaneously study in one sample the expression of thousands of genes (Kurian, (1999) *J Pathol* 187:267-271). A previous hurdle of using this method was the need for micrograms of mRNA or cRNA from samples usually

composed of 10^6-10^7 cells. Here, the novel technology, as described hereinabove, was employed. This allowed the generation of representative cDNA amplificates from a single cell or a low number of cells in quantities sufficient for comprehensive cDNA array hybridization.

10 specimen of each neointimal and quiescent media for the expression of 2,435 genes of known function. While the expression of house-keeping genes was largely comparable between normal and restenotic tissue close to 10 percent of studied genes showed an increased or decreased level of expression. In the present study, it was focused on selected genes that have previously been associated with restenosis. Desmin and mammary-derived growth factor inhibitor (MDGI) expression was selectively downregulated while the expression of prostaglandin G/H synthase-1 (COX-1), thrombospondin-1 (TSP-1), heat-shock protein-70 B (hsp70B) and FK506-binding protein 12 (FKBP12) was found to be upregulated in human neointima hyperplasia. These findings were all confirmed by gene-specific PCR. To study the significance of increased gene expression in neointima, it was investigated whether increased mRNA levels find their reflection in an increased protein level. As exemplified with FKBP12 using immunohistochemistry, it was indeed found a robust overexpression of this regulator of TGF-ß signaling in restenotic tissue. This study shows that cDNA array technology can be used to reliably identify differentially expressed genes in healthy and diseased human tissue even if only very small amounts of material are available.

The in-stent restenosis study group consisted of 13 patients who underwent separate atherectomy procedures by Helix cutter device artherectomy (X-sizer, Endicor) within the renarrowed stent between 4-23 month after primary stent implantation. All patients gave informed consent to the procedure and received 15,000 units heparin before the intervention followed by intravenous heparin infusion, 1,000 units/h for the first 12 h after sheat removal as standard therapy. All patients received aspirin, 500 mg intravenously, before catherisation, and postinterventional antithrombotic therapy consisted of ticlopidine (250 mg bds) and aspirin (100mg bds) throughout the study.

Sample Preparation was carried out as follows:

Atherectomy specimen were immediately frozen in liquid nitrogen after debulking of the lesion, and kept in liquid nitrogen until mRNA preparation was performed as described. For histology and immunhistochemistry of the in-stent restenotic tissue from coronary arteries (n=3), the samples were fixed in 4% paraformaldehyd and embedded in paraffin as described.

The control group consisted of 5 specimen of muscular arteries of the gastrointestinal tract from five different patients and 5 specimen from coronary arteries from three different patients who underwent heart transplantation. The control specimen were immediately frozen in liquid nitrogen. Prior to mRNA preparation, media and intima of the control arteries were prepared and examined for atherosclerotic changes by immunhistochemistry. If there were no atherosclerotic changes of the vessel morphology, the specimen (approx. 1x1 mm) were used as healthy control samples and mRNA and cDNA preparation was performed as described.

For immunohistochemistry of FKBP12, neointima specimen of carotid restenotic arteries (n=2) were obtained by atherectomy and immediately frozen in liquid nitrogen after removal. Three 3 µm serial frozen sections of the samples were mounted onto DAKO ChemMate™ Capillary Gap Microscope slides (100µm).

mRNA Preparation and amplified cDNA was carried out as follows:
Specimen of quiescent vessels or in-stent restenotic tissue were quick-frozen and kept in liquid nitrogen until mRNA preparation and cDNA synthesis was performed. Frozen tissue was ground in liquid nitrogen and the frozen powder dissolved in Lysis/Binding buffer (100mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol (DTT)) and homogenized until complete lysis was obtained. The lysate was centrifuged for 5 min at 10, 000 g at 4° to remove cell debris. mRNA was prepared using the Dynbeads® mRNA Direct Kit™ (Dynal, Germany) following the manufacture's recommendation. Briefly, lysate was added to 50 µL of pre-washed Dynabeads Oligo $(dT)_{25}$ per sample and mRNA was annealed by rotating on a mixer for 30 min at 4°C. Supernatant was removed and Dynabeads Oligo $(dT)_{25}$/mRNA complex was washed twice with washing buffer containing Igepal (50mM Tris-HCl, pH 8.0, 75 mM KCl, 10 mM

DTT, 025% Igepal), and once with washing buffer containing Tween-20 (50mM Tris-HCl, pH 8.0, 75 mM KCl, 10 mM DTT, 0.5% Tween-20).

cDNA was amplified by PCR using the procedure as described hereinabove. First-strand cDNA synthesis was performed as solid-phase cDNA synthesis. Random priming with hexanucleotide primers was used for reverse transcription reaction. mRNAs were each reversely transcribed in a 20 µL reaction volume containing 1x First Strand Buffer (Gibco); 0.01 M DTT (Gibco), 0.25% Igepal, 50 µM CFL5c-Primer [5'-$(CCC)_5$ GTC TAG A $(NNN)_2$-3'], 0.5 mM dNTPs each (MBI Fermentas) and 200 U Superscript II (Gibco), and incubated at 44°C for 45 min. A subsequent tailing reaction was performed in a reaction volume of 10 µL containing 4 mM $MgCl_2$, 0.1 mM DTT, 0.2 mM dGTP, 10 mM KH_2PO_4 and 10 U of terminal deoxynucleotide transferase (MBI Fermentas). The mixture was incubated for 24 min at 37°C.

cDNA was amplified by PCR in a reaction volume of 50 µL containing 1 x buffer 1 (Expand™ Long Template PCR Kit, Boehringer Mannheim), 3% deionized formamide, 1,2 µM CP2-Primer [5'-TCA GAA TTC ATG $(CCC)_5$-3'], 350 µM dNTP and 4.5 U DNA-Polymerase-Mix (Expand™ Long Template PCR Kit, Roche Diagnostics, Mannhein). PCR reaction was performed for 20 cycles with the following cycle parameters: 94°C for 15 sec, 65°C for 0:30 min, 68°C for 2 min; for another 20 cycles with: 94°C for 15 sec, 65°C for 30 sec, 68°C for 2:30 + 0:10/cycle min; 68°C 7 min; 4°C forever.

25 ng of each cDNA was labeled with Digoxigenin-11-dUTP (Dig-dUTP) (Roche Diagnostics) during PCR. PCR was performed in a 50 µL reaction with 1x Puffer 1, 120 µM CP2 primer, 3% deionized formamide, 300 µM dTTP, 350 µM dATP, 350 µM dGTP, 350 µM dCTP, 50 µM Dig-dUTP, 4.5 U DNA-Polymerase-Mix. Cycle parameters were: one cycle: 94°C for 2 min; 15 cycles: 94°C for 15 sec, 63°C for 15 sec, 68°C for 2 min; 10 cycles: 94°C for 15 sec, 68°C for 3 min + 5 sec/cycle; one cycle: 68°C, 7 min, 4°C forever.

Hybridization of Clontech cDNA Arrays with dUTP-labeled cDNA Probes was carried out as follows:

cDNA arrays were prehybridized in DigEASYHyb solution (Roche Diagnostics) containing 50mg/L DNAseI (Roche Diagnostics) digested genomic E. coli DNA, 50mg/L

pBluescript plasmid DNA and 15 mg/L herring sperm DNA (Life Technologies) for 12h at 44°C to reduce background by blocking non-specific nucleic acid-binding sites on the membrane. Hybridization solution was hybridized to commercially available cDNA arrays with selected genes relevant for cancer, cardiovascular and stress response (Clontech). Each cDNA template was denatured and added to the prehybridization solution at a concentration of 5 µg/ml Dig-dUTP-labeled cDNA. Hybridization was performed for 48 hours at 44°C.

Blots were subsequently rinsed once in 2x SSC/0.1% SDS and once in 1x SSC/0.1% SDS at 68°C followed by washing for 15 min once in 0.5x SSC/0.1% SDS and twice for 30min in 0.1x SSC/0.1%SDS at 68°C. For detection of Dig-labeled cDNA hybridized to the array, the Dig Luminescent Detection Kit (Boehringer, Mannheim) was used as described in the user manual. For detection of the chemiluminescence signal, arrays were exposed to chemiluminescence films for 30 min at room temperature. Quantification of array data was performed by scanning of the films and analysis with array vision software (Imaging Research Inc., St. Catharines, Canada). Background was subtracted and signals were normalized to the nine housekeeping genes present on each filter, whereby the average of the housekeeping gene expression signals was set to 1 and the background set to 0. In a pilot study, six clones enriched in one of the two probes were further analyzed by RT-PCR.

Results of the experimental studies are reported as mean expression values of the ten examined specimen of the study or control group. Differences between the two patient groups were analyzed by Wilcoxon-test (SPSS version 8.0). A p-value less than 0.03 was regarded as significant.

A selection of differential hybridization signals were confirmed by PCR using gene-specific primers. PCR reactions were performed using 2.5 ng of each cDNA in 25 µl reaction containing 1x PCR buffer (Sigma), 200 µM dNTPs, 0.1 µM of each primer and 0.75 U Taq Polymerase (Sigma). The following primers were used: desmin, 5'-ACG ATT CCC TGA TGA GGC AG-3' and 5'-CCA TCT TCA CGT TGA GCA GG-3'; thrombospondin-1, 5'-CTG AGA CGC CAT CTG TAG GCG GTG -3' and 5'-GTC TTT GGC TAC CAG TCC AGC AGC-5'; mammary-derived growth inhibitor, 5'- AAG AGA

CCA CAC TTG TGC GG-3' and 5'- AAT GTG GTG CTG AGT CGA GG-5'; prostaglandin G/H synthase-1, 5'- CGG TGT CCA GTT CCA ATA CC-3' and 5'-CCC CAT AGT CCA CCA ACA TG-3'; FKBP12, 5'-ATG CCA CTC TCG TCT TCG AT-3' and 5'-GGA ACA TCA GGA AAA GCT CC-3'; heat shock protein 70B, 5'-TAC AAG GCT GAG GAT GAG GC-3' and 5'-CTT CCC GAC ACT TGT CTT GC-3', and ß-actin, 5'-CTA CGT CGC CCT GGA CTT CGA GC-5' and 5'-GAT GGA GCC GCC GAT CCA CAC GG-3'. PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 µg/ml agarose solution) in TAE buffer (20mM Tris/HCl, 10 mM acetic acid, 1mM EDTA).

Immunohistochemistry was carried out as follows:

Immunohistochemistry for cell typing was performed on paraffin-embedded sections of three neointima specimen from coronary in-stent restenosis and, for detection of FKBP12, on frozen sections of four neointima specimen from carotid restenosis. Three µm serial sections were mounted onto DAKO ChemMate™ Capillary Gap Microscope slides (100 µm) baked at 65°C overnight, deparaffinized and dehydrated according to standard protocols. For antigen retrieval, specimens were boiled 4 min in a pressure cooker in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase was blocked by 1% H_2O_2/methanol for 15 minutes. Unspecific binding of the primary antibody was reduced by preincubation of the slides with 4% dried skim milk in Antibody Diluent (DAKO, Denmark). Immunostaining was performed by the streptavidin-peroxidase technique using the ChemMate Detection Kit HRP/Red Rabbit/Mouse (DAKO, Denmark) according to the manufacturer's description. The procedures were carried out in a DAKO TechMate™ 500 Plus automated staining system. Primary antibodies against smooth muscle actin (M0635, DAKO, Denmark; 1:300), CD3 (A0452, DAKO, Denmark; 1:80), MAC387 (E026, Camon, Germany; 1:20) and FKBP12 (SA-218, Biomol, Germany, 1:20) were diluted in Antibody Diluent and incubated for 1 h at room temperature. After nuclear counterstaining with hematoxylin, the slides were dehydrated and coverslipped with Pertex (Medite, Germany).

For FKBP12 immunhistochemistry, 3 µm frozen, serial sections of the neointima specimen from carotid restnosis were mounted onto DAKO ChemMate™ Capillary Gap Microscope slides (100 µm).

The following results were obtained:

(a) *The Cellular Composition of Debulked In-stent Restenotic Material*

Representative samples obtained from x-sizer treatment of a neointimal hyperplasia were analyzed by immunhistochemistry in order to determine its cellular composition. The restenotic tissue analyzed was removed by x-sizer debulking from coronary arteries more than two month after PTCA and stent implantation. The amount of tissue generated by this procedure was very low containing an estimated 300 - 10000 cells. Figure 7A shows an E.-van-Giesson staining of a section cut from a small sample of debulked restenotic material. With this staining procedure, collagen fibers stain red, fibrin stains yellow and cytoplasm of smooth muscle cells stains dark-yellow-brown. The majority of the volume of debulked material was composed of loose extracellular matrix-like collagen fibers stained in light red. Yellow fibrin staining was barely detectable. Cells with spindle-shaped nuclei and a yellow/brown-stained cytoplasm were frequent. Their identity as smooth muscle cells and their high abundance in restenotic material was supported by immunostaining with an antibody against smooth muscle α-actin (Fig. 7B). There, the staining pattern of a section from an entire specimen as used for gene expression analysis is shown. As described below, such samples also gave raise to a strong smooth muscle-specific α-actin mRNA signal (see Figs. 8). These results support findings from previous studies (Komatsu, (1998), *Circulation* 98:224-233; Strauss (1992), *J. Am. Coll. Cardiol.* 20:1465-1473; Kearney (1997), *Circulation* 95:1998-2002) demonstrating that the main cell type found in neointima is derived from smooth muscle cells. As described in the literature, mononuclear infiltrates in some areas of debulked restenotic tissue specimen could also be identified (data not shown). These infiltrates consisted mainly of macrophages and to a lesser degree of t-lymphocytes. No b-lymphocytes were detectable in the restenotic tissue by using an antibody against CD20 for immunhistochemical staining (data not shown).

(b) *Expression of Specific Genes in Microscopic Human Tissue Samples*

In order to optimally preserve the *in situ* mRNA levels, restenotic and control specimen were immediately frozen after harvest in liquid nitrogen and carefully lyzed as described hereinabove. After PCR amplification of the synthesized cDNA the amount of the amplified cDNA was measured by a dot blot assay and found to be between 200-300 ng/µl. The quality of every amplified cDNA sample was tested by gene-specific PCR using primers detecting cDNAs for ß-actin, smooth muscle cell α-actin and the ubiquitous elongation factor EF-1α. Figure 8 shows a representative result with material from patient B and control media from donor b. In both specimen, PCR signals of the correct size from house-keeping genes ß-actin and EF-1α were detectable in equivalent amounts (compare lanes 1 and 2 with lanes 4 and 5). Additionally, α-actin signals as marker for smooth muscle cells was obtained from each specimen (lanes 3 and 6). These results show that mRNA preparation, cDNA synthesis and PCR amplification of cDNA is feasible with microscopic human restenosis samples..

(c) *Comparative Gene Expression Profiling Using Microscopic Human Tissue Samples*

To identify differentially expressed mRNAs in restenotic versus healthy specimen, the cDNAs was labeled during PCR amplification with digoxigenin-labeled dUTP as described hereinabove. This label allows for a highly sensitive, chemiluminescence-based detection of hybridization signals of cDNA arrays on photographic films. The nylon filters with cDNA arrays were pre-hybridized with DNAseI-digested genomic E. coli DNA and with DNAseI-digested pBluescript plasmid DNA. This procedure was employed to maximally reduce non-specific DNA binding to the array. Each labeled probe was hybridized to three different commercial cDNA arrays which allowed for the expression analysis of a total of 2,435 known genes. Figure 9 shows a representative hybridization pattern obtained with one array using probes prepared from restenotic tissue of patient B (panel A) and media of donor b (panel B). Consistent with the gene-specific analysis shown in Figure 8, comparable hybridization signals were obtained with the positive control of human genomic cDNA spotted on the right and bottom lanes of the array and with cDNA spots of various housekeeping genes (see for

instance, spots D). If a biological specimen was omitted from cDNA synthesis and PCR amplification reactions almost no hybridization signals were obtained (Fig. 9, panel C), showing that hybridization signals were almost exclusively derived from added samples and not from DNA contaminations in reagents or materials used.

Visual inspection of the hybridization patterns readily identified a number of signals that are different between healthy and diseased tissue (for instance signals A, B and C in Figs. 9A and B). Samples from restenotic tissues consistently gave more signals than control tissues. Hybridization signals obtained from the use of three different cDNA arrays with 10 restenosis patient samples and 10 normal media samples were quantitated by densitometric analysis of photographic films and the data electronically compiled and further analyzed for statistics. Expression levels for 53 out of 2,435 genes is shown in Figure 10 whereby one grey value corresponds to the signal intensity as shown in the figure legend. A considerable variation of gene expression is evident for most genes shown which may reflect genetic and physiological differences of patients and donors. For further analysis and verification by gene-specific PCR, only genes were considered that showed a differential expression with a statistical difference of at least p=0.03 by the Wilcoxon Test. Six such genes are highlighted in the list (Fig. 10). A total of 224 genes out of 2435 known genes was found to be differentially regulated in neointima with high statistical significance. Their comprehensive in-depth analysis will be published elsewhere. Indicative for a comparable sample quality, eight housekeeping genes showed very similar hybridization signal intensities with all 20 samples (Fig. 10, bottom).

(d) *Validation of cDNA Array Data by Gene-specific PCR*

Out of the list depicted in Figure 10, six differentially regulated genes and one housekeeping gene were selected for validation of hybridization signals through PCR using gene-specific primers. All PCR signals obtained had the predicted size. In support of an equal quality of samples, the ß-actin signal (bottom) showed a very similar intensity with all 20 samples. By comparing gene-specific PCR signals (Fig. 11) with hybridization signals obtained from cDNA arrays (Fig.

11) it was found that 135 out of 140 signals matched with respect to intensity. This corresponds to a 96% fidelity of hybridization signals from cDNA arrays showing that the here employed gene expression profiling approach is comparable with respect to quality and sensitivity to gene-specific PCR.

(e) Aberrant Gene Expression in Human Restenotic Tissue

Desmin, a mesenchymal marker, was found strongly expressed in the control media, whereas only weak signals were found in the restenotic specimen (Figs. 10 and 11). Desmin is a marker for SMCs that is highly expressed in quiescent, differentiated SMCs. Its expression is reduced in de-differentiated, proliferating SMCs, e.g., in SMCs of atherosclerotic plaques (Ueda (1991), *Circulation* 83:1327-1332). Downregulation of desmin in restenotic tissue implies that the spindle-shaped cells in the restenotic material are de-differentiated, proliferating SMCs. Inversely, TSP-1, an extracellular matrix protein, that is important in TGF-ß activation and SMC migration and proliferation (Yehualaeshet (1999), *Am J Pathol* 155:841-851; Scott (1988), *Biochem.Biophys.Res.Commun.* 150:278-286), is markedly upregulated in the majority of neointimal specimen versus the control samples. The COX-1, stress-induced hsp70B and the ubiquitously expressed FKBP12 genes were significantly upregulated in almost all neointimal hyperplasia and barely, if at all, expressed in control specimen (Figs. 10 and 11). The tumor suppressor MDGI was strongly expressed in quiescent smooth muscle whereas little expression was found in a few neointima hyperplasia samples.

None of the restenotic lesions expressed desmin (0/0) compared to 100% of controls (10/10), only 30% (3/10) of the neointimal specimen expressed MDGI very slightly, whereas it was highly expressed in 8/10 (80%) of the controls. Otherwise, TSP-1 (7/10), COX-1 (9/10), hsp70B (8/10) and FKBP12 (10/10) were significantly upregulated in neointimal versus control specimen (TSP-1 [0/10], hsp70B [0/10], COX-1 [0/10], FKBP12 [1/10]).

(f) FKBP12 Protein Expression Is Upregulated in Human Restenotic Tissue

Upregulation of mRNA levels does not stringently indicate an increased level of protein. Among the genes that were found to be upregulated in human neointima,

FKBP12 is particularly interesting since it is a regulator of TGF-β signaling and target for the drugs FK506 and rapamycin. A therapeutic effect of rapamycin in rodent models (Gallo (1999), *Circulation* 99:2164-2170) of restenosis is poorly understood but may be related to changes in the expression level of FKBP12. Using an antibody specific for FKBP12, human restenotic tissue from carotid restenosis (n=3) was analyzed and control tissue (n=3) for the expression of the protein. As shown in Figure 12, an increase in FKBP12 protein in the cytoplasm of SMCs from restenotic lesions as identified by their spindle-shaped nuclei was detected (Fig. 12B and D). Whereas no FKBP12 was detectable in control SMCs of healthy media (Fig. 12C), a distinct staining in SMCs of neointima was found (Fig. 12D). Interestingly, especially smooth muscle cells lying in the border zone between neointima and healthy media of restenotic vessels expressed high levels of the FKBP12 protein (Fig. 11B).

Example VI: Characterization of the transcriptome of human restenotic tissue

The expression of 2,435 genes of known function (see Example V) was investigated in atherectomy specimen of 10 patients with in-stent restenosis, blood cells of 10 patients, normal coronary artery specimen of 11 donors, and cultured human coronary artery smooth muscle cells. 224 genes that were differentially expressed with high statistical significance ($p<0.03$) between neointima and control tissue which could be grouped as follows: (1) genes only expressed in neointima; (2) genes expressed in both neointima and proliferating smooth muscle cells; (3) genes expressed in both neointima and blood samples; and (4) genes expressed in control tissue but barely in neointima. The transcriptome of human neointima showed significant changes related to proliferation, apoptosis, inflammation, cytoskeletal reorganization and tissue remodeling. Furthermore, in neointima 32 upregulated genes were identified that are related to interferon-γ signaling.

In the present study, 10 specimen of neointimal and 11 specimen of quiescent intima/media for the expression of 2,435 human genes of known function were analyzed. While the expression of housekeeping genes was largely comparable

between normal and restenotic tissue, an impressive number of genes (n=224) showed an increased or decreased level of expression. The gene expression pattern in neointima showed the anticipated proliferative response with induction of genes mainly expressed in G1/S phase, changes of the smooth muscle phenotype from contractile to synthetic SMCs and changes in synthesis of extracellular matrix proteins. Additionally, a pro-inflammatory expression pattern characterized by the presence of markers for macrophages and T lymphocytes and by the expression of numerous genes with known functions in the cellular response to IFN-γ were observed. The IRF-1 protein, a pivotal transcription factor in IFN-γ signaling, was found overexpressed in SMCs of human neointima.

The clinical characteristics of the patients of the study group of this Example are presented in Table 7.

Table 7

Clinical Data of 13 Patients

Patient	Age, y	Sex	Indication for Stent	Stent Site	Interval/Stent/Poststent Restenosis	Interval Stent/Debulking	Smoker	Hypercholesterolemia	Arterial Hypertension	Diabetes mellitus	Multivessel disease	familial risk
1	77	m	AMI	RCA	5 m	11 m	-	+	+	+	+	-
2	62	m	SAP	LAD	6 m	19 m	-	+	+	-	-	+
3	57	m	ISAP	ACVB 7	4 m	10 m	+	-	+	+	+	-
4	68	m	failed Bypass	ACVB 14	4 m	4 m	-	+	+	-	+	-
5	80	m	AMI	LAD	7 m	7 m	-	+	+	-	+	-
6	67	m	Restenosis	RCA	12 m	23 m	-	+	+	-	+	-
7	44	f	Restenosis after PTCA	RCA	3 m	8 m	-	+	7	-	-	-
8	75	m	Restenosis after PTCA	RCA	6 m	6 m	-	+	-	-	+	-
9	86	m	Restenosis after PTCA	RCA	5 m	5 m	-	+	+	-	-	-
10	44	m	Restenosis after PTCA	LAD	6 m	6 m	+	+	+	-	-	+
11	76	m	AMI	LAD	6 m	6 m	-	+	+	-	-	-
12	46	m	Restenosis after PTCA	LAD	5 m	5 m	-	+	+	-	-	+
13	69	m	Restenosis after PTCA	LAD	4 m	16 m	-	+	+	-	+	-

All atherectomy specimen were immediately frozen in liquid nitrogen after debulking of the lesion, and kept in liquid nitrogen until mRNA preparation was performed as described above.

The control group consisted of 5 specimen of muscular arteries of the intestine from five patients and 6 specimen from coronary arteries from three patients who underwent heart transplantation. The control specimen were immediately frozen in liquid nitrogen. Prior to mRNA preparation, media and intima of the arteries were prepared. A small piece of the specimen (approx. 1 mm^3) was immediately lysed, whereas the rest was histologically examined for atherosclerotic changes. If there were no atherosclerotic changes of vessel morphology detectable, the specimen were used as "healthy" control samples and mRNA and cDNA preparation was performed as described.

The neointimal tissue of carotid (n=3) and femoralis (n=3) arteries was generated by atherectomy within the restenosis and immediately frozen after removal in liquid nitrogen. For histologic evaluation and immunohistochemistry of the in-stent restenotic tissue from coronary arteries (n=3) and of the neointima of restenotic peripheral arteries (n=6), the samples were fixed in 4% paraformaldehyd and embedded in paraffin as described.

Blood samples were obtained immediately after revascularization of the restenotic vessel. Eight ml blood samples were collected into 35 ml of TriReagent Blood (MBI Fermentas, Germany) and subsequently frozen at –80°C until RNA preparation was performed as described in the manufacture's protocol. 1µg of total RNA of blood cells were dissolved in 1000 µL Lysis/Binding buffer and mRNA and cDNA synthesis was prepared as described above.

Cell Culture was carried out as follows:

Primary human coronary artery smooth muscle cells (CASMCs) were obtained from CellSystems (St. Kathrinen, Germany) and were grown in Smooth Muscle Cell Growth Medium (CellSystems, St. Kathrinen, Germany) containing 5% fetal calf serum (CellSystems, St. Kathrinen, Germany) at 37°C in a humidified atmosphere of 5% CO_2. CASMCs were used in experiments between passages 2 and 4. For cDNA synthesis of proliferating CASMCs were washed three times with ice-cold phosphate-buffered saline

and $1x10^4$ cells were subsequently lysed in 1000µL Lysis/Binding puffer before mRNA was prepared as described above.

Determination of Gene Expression Patterns was carried out as follows:
Sample mRNA preparation, cDNA synthesis, PCR amplification and probe labeling, cDNA array hybridization and data analysis were performed as described hereinabove, in particular in Example V. The obtained cDNA probes were hybridized to Human 1.2, Cancer 1.2, Cardiovascular and Stress cDNA arrays (Clontech, Heidelberg, Germany) with a total of 2,435 genes of known function. There was an approximately 20% redundancy of genes among cDNA arrays. For analysis of microscopic human tissue samples down to a single cell level the here described new method of cDNA synthesis and PCR amplification was used (see Examples I to V).

Quantification of array data was performed by scanning of the films and analysis with array vision software (Imaging Research Inc., St. Catharines, Canada). Background was subtracted and signals were normalized to the nine housekeeping genes present on each filter, whereby the average of the housekeeping gene expression signals was set to 1 and the background set to 0. For the logarithmic presentation shown in Figure 1, values were multiplied by 1000. A mean value $\geq$0,05 in the average of all samples in one group was regarded as a positive signal. Differences in the mean expression level by a factor $\geq$2.5-fold between the study and the control group were further statistically analyzed.

Results of the experimental analysis are given as mean expression values of the ten examined specimen of the study group or the eleven examined specimen of the control group. Differences between the patient and donor groups were analyzed by the Wilcoxon-test (SPSS version 8.0). Genes were only considered to be differentially expressed between the two groups if their p-values in the Wilcoxon test were <0.03, and if a differential expression was observed in at least 5 out of 10 samples within one study group, while there was 0 out of 10 within the other group; or at least 7 out of 10 samples within one group, while there were maximally 3 out of 10 within the other group.

Immunhistochemistry was carried out as follows:

Immunhistochemistry was performed on paraffin-embedded sections from 3 neointima specimen from coronary in-stent restenosis, 3 neointima specimen from A. femoralis and 3 neointima specimen from carotid neointima specimen. Three µm serial sections were mounted onto DAKO ChemMate™ Capillary Gap Microscope slides (100µm), baked at 65°C overnight, deparaffinized and dehydrated according to routine protocols. For antigen retrieval, specimen were boiled 4 minutes in a pressure cooker in citrate buffer (10 mMol, pH 6.0). Endogenous peroxidase was blocked by 1% H_2O_2/methanol for 15 minutes. Unspecific binding of the primary antibodies was reduced by preincubation of the slides with 4% dried skim milk in Antibody Diluent (DAKO, Denmark). Immunostaining was performed by the streptavidin-peroxidase technique using the Dako ChemMate Detection Kit HRP/Red Rabbit/Mouse (DAKO Denmark) according to the manufacturers description. The procedures were carried out in a DAKO TechMate™ 500 plus automated staining system. Primary antibodies against smooth muscle actin (M0635, DAKO, Denmark; 1:300), CD3 (A0452, DAKO, Denmark; 1:80), MAC387 (E026, Camon, Germany; 1:20) and IRF-1 (sc-497, Santa Cruz, U.S.A.) were diluted in Antibody Diluent and incubated for 1 h at room temperature. After nuclear counterstaining with hematoxylin, slides were dehydrated and coverslipped with Pertex (Medite, Germany).

The following results were obtained:

(a) *Differential Gene Expression in Human Neointima*

A total of 1,186 genes (48.7%) out of 2,435 yielded detectable hybridization signals on cDNA arrays with neointima and control samples over a 20-fold range of expression level (Fig. 13A) Thereof 352 genes (14.5%) appeared to be differentially expressed by a factor >2.5-fold between restenotic and control samples. However, expression levels considerably varied among individual samples (see, e.g., Fig. 15). Therefore, a statistical analysis was employed to identify those genes that are differentially expressed between study and control groups with high significance (see Methods). This way, 224 genes (9.6%) were identified that were differentially expressed by a factor of at least 2.5-fold between the restenosis study group and the control group with a significance in

the Wilcoxon test of $p<0.03$. 167 (75%) genes thereof were found overexpressed and 56 genes (25%) underexpressed in the restenosis study group compared to the control group (Fig. 13B).

In addition to the statistical significance, the validity of expression data was supported by a 20% redundancy of cDNA elements on the four arrays used. This way, a substantial number of hybridization signals was determined in duplicate or triplicate in independent hybridization experiments. Four examples of duplicate determinations are shown in Fig. 16 (top) which all showed a high degree of reproducibility. As a further validation of hybridization signals, 38 of the differentially expressed genes were selected for PCR analysis of cDNA samples using gene-specific primers. Hybridization signals for 35 (92%) out of 38 genes could be verified by gene-specific PCR yielding signals of the predicted size and relative quantity (data not shown). These data shows that the employed cDNA array approach is comparable with respect to quality and sensitivity to gene-specific PCR. Lastly, among the 224 aberrantly expressed genes in neointima 112 have previously been described in the literature as being expressed in neointima, SMCs, fibroblasts, endothelial cells or mesenchym (Fig. 14 marked with '#').

With respect to neointima expression, the 224 aberrantly regulated genes fell into four subgroups (Fig. 14). Group I lists 62 genes that were overexpressed in neointima and not highly or detectably expressed in control vessels, CASMCs or blood cells (Fig. 14A). In group II, 43 genes are listed that are similarly expressed in neointima and CASMCs, suggesting that this gene cluster in neointima was contributed by proliferating SMCs (Fig. 14B). In group III, 62 genes are listed that are similarly expressed in neointima and blood cells, suggesting that this gene cluster was contributed to that of neointima by infiltrated blood cells (Fig. 14C). This notion is supported by the expression in group III of the largest number of genes related to inflammation in all four groups. Lastly, in group IV, 56 genes are listed that are downregulated in neointima compared to the control group (Fig. 14D). In the following, the aberrant expression of selected genes in neointima will be discussed in the context of gene function.

In summary, the following differentially expressed genes have been detected in human neointima:

Gene Name	GenBank Accession #	SwissProt Accession #
80-kDa nuclear cap-binding protein	D32002	Q09161
activator 1 140-kDa subunit (A1 140-kDa subunit); replication factor C large subunit; DNA-binding protein PO-GA	L14922	P35251
activator 1 37-kDa subunit; replication factor C 37-kDa subunit (RFC37); RFC4	M87339	P35249
adenylate kinase isoenzyme 1 (AK1); ATP-AMP transphosphorylase; myokinase	J04809	P00568
adipocyte fatty acid-binding protein 4 (FABP4; AFABP); adipocyte lipid-binding protein (ALBP)	J02874	P15090
allograft inflammatory factor 1 (AIF1); ionized calcium-binding adapter molecule 1	U19713	P55008
alpha-1-antitrypsin precursor; alpha-1 protease inhibitor; alpha-1-antiproteinase	X02920	P01009
alpha-2-antiplasmin	D00174	P08697
alpha-2-macroglobulin precursor (alpha-2-M)	M11313	P01023
alpha-galactosidase A precursor; melibiase; alpha-D-galactoside galactohydrolase	X05790	P06280
amiloride-sensitive epithelial sodium channel beta subunit; nonvoltage-gated sodium channel 1 beta subunit (SCNEB; beta NACH); SCNN1B	X87159	P51168
angiotensinogen precursor (AGT)	K02215	P01019
apolipoprotein E precursor (APOE)	M12529	P02649
atrial natriuretic peptide receptor B precursor (ANPB; NPRB); guanylate cyclase B (GCB)	L13436	P20594
autosomal dominant polycystic kidney disease II (PKD2)	U50928	Q13563

B-cell-associated molecule CD40	X60592	P25942
BCL-2 binding athanogene-1 (BAG-1); glucocorticoid receptor-associated protein RAP46	S83171; Z35491	Q99933
BCL-2-related protein A1 (BCL2A1); BFL1 protein; hemopoietic-specific early response protein; GRS protein	U29680; Y09397	Q16548; Q99524
BIGH3	M77349	Q15582
bikunin; hepatocyte growth factor activator inhibitor 2	U78095	O00271; O43291
brain glucose transporter 3 (GTR3)	M20681	P11169
brain-specific polypeptide PEP-19; brain-specific antigen PCP-4	U52969	P48539
Bruton's tyrosine kinase (BTK); agammaglobulinaemia tyrosine kinase (ATK); B-cell progenitor kinase (BPK)	U10087; X58957	Q06187
C5a anaphylatoxin receptor (C5AR); CD88 antigen	M62505	P21730
cadherin 16 (CDH16); KSP-cadherin	AF016272	P75309
calcium & integrin-binding protein (CIB)	U85611	Q99828
carboxypeptidase H precursor (CPH); carboxypeptidase E (CPE); enkephalin convertase; prohormone processing carboxypeptidase	X51405	P16870
carboxypeptidase N	X14329	P15169
caspase-8 precursor (CASP8); ICE-like apoptotic protease 5 (ICE-LAP5); MORT1-associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease (FADD-like ICE; FLICE); apoptotic cysteine protease MCH-5	U60520; U58143; X98172; AF00962	Q14790; Q15780
caveolin 3	AF043101	P56539
CBL-B	U26710	Q13191

CDC42 homolog; G25K GTP-binding protein (brain isoform + placental isoform)	M35543 + M57298	P21181 + P25763
cell surface adhesion glycoproteins LFA-1/CR3/p150,95.beta-subunit precursor; LYAM1; integrin beta 2 (ITGB2); CD18 antigen; complement receptor C3 beta subunit	M15395	P05107; Q16418
cell surface glycoprotein mac-1 alpha subunit precursor; CD11B antigen; leukocyte adhesion receptor MO1; integrin alpha M (ITGAM); neutrophil adherence receptor alpha M subunit; CR3A	J04145	P11215
cell surface glycoprotein MUC18; melanoma-associated antigen A32; CD146 antigen; melanoma adhesion molecule	M28882	P43121
C-fgr proto-oncogene (p55-FGR); SRC2	M19722	P09769
c-fos proto-oncogene; G0S7 protein	K00650	P01100
chemokine receptor-like 2; IL8-related receptor DRY12; flow-induced endothelial G protein-coupled receptor (FEG1); G protein-coupled receptor GPR30; GPCR-BR)	AF015257	Q99527; Q99981; O00143; Q13631
clone 23815 (Soares library 1NIB from IMAGE consortium)	U90916	none
coagulation factor XII	M11723	P00748
collagen 16 alpha 1 subunit precursor (COL16A1)	M92642	Q07092
collagen 18 alpha 1 subunit (COL18A1)	L22548	P39060
collagen 6 alpha 1 subunit (COL6A1)	X15880	P12109
collagen 6 alpha 2 subunit (COL6A2)	M34570	Q13909; Q13911
coronin-like protein P57	D44497	P31146

c-src kinase (CSK); protein-tyrosine kinase cyl	X59932	P41240
cyclin-dependent kinase 4 inhibitor (CDK4I; CDKN2); p16-INK4; multiple tumor suppressor 1 (MTS1)	L27211	P42771; Q15191
cyclin-dependent kinase inhibitor 1 (CDKN1A); melanoma differentiation-associated protein 6 (MDA6); CDK-interacting protein 1 (CIP1); WAF1	U09579; L25610	P38936
cytidine deaminase (CDA)	L27943	P32320
death-associated protein 1 (DAP1)	X76105	P51397
desmin (DES)	U59167	P17661; Q15787
DNAX activation protein 12	AF019562	O43914
dual-specificty A-kinase anchoring protein 1	X97335	Q92667
early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225	X52541; M62829	P18146
early response protein NAK1; TR3 orphan receptor	L13740	P22736
endothelial differentiation gene 1 (EDG1)	M31210; AF022137	P21453
endothelin 2 (ET2)	M65199	P20800
ephrin A receptor 4 precursor; tyrosine-protein kinase receptor sek; hek8	L36645	P54764
epithelial discoidin domain receptor 1 precursor (EDDR1; DDR1); cell adhesion kinase (CAK); TRKE; RTK6		X74979
estradiol 17 beta-dehydrogenase 1	M36263	P14061

estrogen-related receptor alpha	X51416; Y00290	P11474
ets domain protein elk-3; NET; SRF accessory protein 2 (SAP2)	Z36715	P41970
extracellular superoxide dismutase precursor (EC-SOD; SOD3)	J02947	P08294
farnesyltransferase beta	L10414	P49356
FC-epsilon-receptor gamma subunit	M33195	P30273
FK506-binding protein (FKBP; FKBP12); peptidyl-prolyl cis-trans isomerase (PPIASE); rotamase	M34539; M80199; M80706; M92423; J05340; X55741; X52220	
fli-1 oncogene; ergB transcription factor	M93255	Q01543
FMLP-related receptor I (FMLPRII); RMLP-related receptor I (RMLPRI)	M76673	P25089
focal adhesion kinase 2 (FADK2; FAK2); cell adhesion kinase beta (CAKbeta); proline-rich tyrosine kinase 2 (PYK2)	L49207 + U43522 + U33284	Q14289; Q16709; Q13475
frizzled-related FrzB (FRITZ) + FrzB precursor + frezzled (FRE)	U91903 + U24163 + U68057	O00181 + Q92765 + Q99686
G protein-coupled receptor EDG4	AF011466	O43431
G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	X59798; M64349	P24385
G1/S-specific cyclin D3 (CCND3)	M92287	P30281
gamma-interferon-inducible protein; IP-30	J03909	P13284
GAP junction alpha-1 protein	X52947	P17302

glutathione-S-transferase (GST) homolog	U90313	P78417
glycerol kinase	L13943	P32189
G-protein-coupled receptor HM74	D10923	P49019
granulocyte colony stimulating factor receptor precursor (GCSF-R); CD114 antigen	M59818	Q99062
granulocyte-macrophage colony-stimulating factor receptor alpha (GM-CSFR-alpha); CSW116 antigen	X17648	P15509
growth arrest & DNA-damage-inducible protein 45 beta (GADD45 beta)	AF078077	none
growth arrest & DNA-damage-inducible protein 45 gamma (GADD45 gamma)	AF078078	none
growth factor receptor-bound protein 2 (GRB2) isoform; GRB3-3; SH2/SH3 adaptor GRB2; ASH protein + epidermal growth factor receptor-bound protein 2 (EGFRBP-GRB2)	L29511; M96995	P29354
growth inhibitory factor; metallothionein-III (MT-III)	D13365; M93311	P25713
GTP-binding protein ras associated with diabetes (RAD1)	L24564	P55042
guanine nucleotide-binding protein G(Y) alpha 11 subunit (GNA11; GA11)	M69013	P29992; Q14350; O15109
heart fatty acid-binding protein 3 (FABP3; HFABP); muscle fatty acid-binding protein (MFABP); mammary-derived growth inhibitor (MDGI)	Y10255	P05413; Q99957
heat shock 70-kDa protein 6 (heat shock 70-kDa protein B)	X51757; M11236	P48741
heat shock cognate 71-kDa protein	Y00371	P11142
heme oxygenase 1 (HO1); HSOXYGR	X06985	P09601
high mobility group protein (HMG-I)	M23619	P17096

high-affinity interleukin-8 receptor A (IL-8R A); IL-8 receptor type 1; CDW128	M68932	P25024
high-affinity nerve growth factor receptor precursor; trk-1 transforming tyrosine kinase protein; p140-TRKA; p68-trk-T3 oncoprotein	X03541	P04629
histone H4	X67081	none
HLA class II histocompability antigen alpha subunit precursor (MHC-alpha)	M31525	P06340
homeobox protein HOXB7; HOX2C; HHO.c1	M16937	P09629
hormone-sensitive lipase		Q05469
hydroxyacyl-CoA dehydrogenase; 3-ketoacyl-CoA thiolase; enoyl-CoA hydratase beta subunit	D16481	P55084
IgG receptor FC large subunit P51 precursor (FCRN); neonatal FC receptor; IgG FC fragment receptor transporter alpha chain	U12255	P55899
IMP dehydrogenase 1	J05272	P20839
insulin receptor precursor (INSR)	M10051; X02160	P06213
insulin-like growth factor binding protein 6 precursor (IGF-binding protein 6; IGFBP6; IBP6)	M62402	P24592
insulin-like growth factor I receptor (IGF1R)	X04434; M24599	P08069
integrin alpha 3 (ITGA3); galactoprotein B3 (GAPB3); VLA3 alpha subunit; CD49C antigen	M59911	P26006
integrin alpha 7B precursor (IGA7B)	X74295	Q13683
integrin alpha 8 (ITGA8)	L36531	P53708
integrin beta 7 precursor (ITGB7)	M62880; S80335	P26010
inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4); plasma kallikrein-sensitive glycoprotein 120 (PK-120)	D38595	Q14624
intercellular adhesion molecule 2 precursor (ICAM2); CD102 antigen	X15606	P13598

Intercellular adhesion molecule 3 precursor (ICAM3); CDW50 antigen; ICAM-R	X69711; X69819	P32942
Intercellular adhesion molecule-1 precursor (ICAM1); major group rhinovirus receptor; CD54 antigen	J03132	P05362
Interferon regulatory factor 1 (IRF1)	X14454	P10914
Interferon regulatory factor 7 (IRF-7)	U73036	Q92985
Interferon-gamma (IFN-gamma) receptor beta subunit precursor; IFN-gamma accessory factor 1 (AF1); IFN-gamma transducer 1 (IFNGT1)	U05875	P38484
Interferon-gamma receptor (IFNGR)	A09781	none
Interferon-induced 56-kDa protein (IFI-56K)	X03557	P09914
Interferon-inducible protein 9-27	J04164	P13164
Interleukin-1 beta convertase precursor (IL-1BC); IL-1 beta converting enzyme (ICE); p45; caspase-1 (CASP1)	U13699; M87507; X65019	P29466
Interleukin-1 receptor type II precursor (IL-1R2); IL-1R-beta	X59770	P27930
Interleukin-16 (IL-16); lymphocyte chemoattractant factor (LCF)	M90391	Q14005
Interleukin-2 receptor gamma subunit (IL-2R gamma; IL2RG); cytokine receptor common gamma chain precursor; p64	D11086	P31785
Interleukin-6 receptor alpha subunit precursor (IL-6R-alpha; IL6R); CD126 antigen	M20566; X12830	P08887
I-rel (RELB)	M83221	Q01201
leukocyte IgG receptor (FC-gamma-R)	J04162	P08637
lipoprotein-associated coagulation inhibitor	J03225	P10646
low affinity immunoglobulin gamma FC receptor II-A precursor (FC-gamma RII-A; FCRII-A; IgG FC receptor II-A); CD32 antigen	M31932	P12318

low-density lipoprotein receptor-related protein LR11 precursor	Y08110	Q92673
L-selectin precursor; lymph node homing receptor (LNHR); leukocyte adhesion molecule 1 (LAM1) leukocyte surface leu-8 antigen; GP90-MEL; leukocyte-endothelial cell adhesion molecule 1 (LECAM1); CD62L antigen; SELL	M25280	P14151
LUCA2; lysosomal hyaluronidase 2 (HYAL2) ; PH-20 homolog	U09577	Q12891
lymphocyte antigen	M81141	Q30099
lymphoid-restricted homolog of SP100 protein (LYSP100)	U36500	Q13342
lymphotoxin-beta (LT-beta; LTB); tumor necrosis factor C (TNFC)	L11015	Q06643
lysosomal acid lipase/cholesteryl ester hydrolase precursor (LAL); acid cholesteryl ester hydrolase; sterol esterase; lipase A (LIPA); cholesteryl esterase	M74775	P38571
lysosomal pro-X carboxypeptidase	L13977	P42785
macrophage colony stimulating factor I receptor precursor (CSF-1-R); fms proto-oncogene (c-fms); CD115	X03663	P07333
macrosialin precursor	S57235	P34810
manic fringe	U94352	O00587
matrix metalloproteinase 17 (MMP17); membrane-type matrix metalloproteinase 4 (MT-MMP4)	X89576	Q14850
matrix metalloproteinase 9 (MMP9); gelatinase B; 92-kDa type IV collagenase precursor (CLG4B)	J05070; D10051	P14780
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	M20430	Q30166
microsomal aminopeptidase N; myeloid plasma membrane glycoprotein CD13	M22324	P15144
microtubule-associated protein 1B	L06237	P46821

migration inhibitory factor-related protein 14 (MRP14); calgranulin B; leukocyte L1 complex heavy subunit; S100 calcium-binding protein A9	X06233	P06702
migration inhibitory factor-related protein 8 (MRP8); calgranulin A; leukocyte L1 complex light subunit; S100 calcium-binding protein A8; cystic fibrosis antigen (CFAG)	X06234	P05109
myeloid cell nuclear differentiation antigen (MNDA)	M81750	P41218
myotonin-protein kinase; myotonic distrophy protein kinase (MDPK); DM-kinase (DMK)	L19268	Q09013
neurogenic locus notch protein (N)	M99437	Q04721
neurogranin (NRGN); RC3	Y09689	Q92686
neurotrophic tyrosine kinase receptor-related 3; TKT precursor	X74764	Q16832
neutrophil cytosol factor 2; neutrophil NADPH oxidase factor 1 (NCF1); p47-PHOX); 47-kDa autosomal chronic granulomatous disease protein	M25665	P14598
neutrophil gelatinase-associated lipocalin precursor (NGAL); 25-kDa alpha-2-microglobulin-related subunit of MMP9); lipocalin 2; oncogene 24P3	X99133	P80188
ninjurin-1	U72661	Q92982
NKG5 protein precursor; lymphokine LAG2; T-cell activation protein 519	X54101	P22749
NT-3 growth factor receptor precursor (NTRK3); C-trk tyrosine kinase (TRKC)	U05012	Q16288; Q16289; Q12827
nuclear receptor-related 1	X75918	P43354
NuMA	Z11583	Q14981
osteoclast stimulating factor	U63717	Q92882

P126 (ST5)	U15131	P78524
P2X purinoceptor 1; ATP receptor P2X1	X83688	P51575
P2X purinoceptor 5 (P2X5)	AF016709	Q93086
paxillin	U14588	P49023
PC8 precursor	U33849	Q16549
peripheral myelin protein 22 (PMP22); CD25 protein; SR13 myelin protein	D11428	Q01453
peroxisomal bifunctonal enzyme	L07077	Q08426
phenol-sulfating phenol sulfotransferase 1 (PPST1); thermostable phenol sulfotransferase (TS-PST); HAST1/HAST2; ST1A3; STP1 + PPST2; ST1A2; STP2 + monoamine-sulfating phenol sulfotransferase	U09031 + U28170 + L19956	P50225 + P50226 + P50224
phospholipase C beta 2 (PLC-beta 2; PLCB2); 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta 2	M95678	Q00722
phosphoribosyl pyrophosphate synthetase subunit I	D00860	P09329
PIG7	AF010312	Q99732
pim-1 proto-oncogene	M54915	P11309
platelet basic protein precursor (PBP); connective tissue activating peptide III (CTAP III); low-affinity platelet factor IV (LA PF4); beta thromboglobulin (beta TG); neutrophil activating peptide 2 (NAP2)	M54995; M38441	P02775
platelet endothelial cell adhesion molecule	HS78146	P16284
platelet membrane glycoprotein IIB precursor (GP2B); integrin alpha 2B (ITGA2B); CD41 antigen	M34480; J02764	P08514

platelet membrane glycoprotein IIIA precursor (GP3A); integrin beta 3 (ITGB3); CD61 antigen	J02703; M25108	P05106; Q13413; Q16499
platelet-activating factor receptor (PAFR)	D10202	P25105
platelet-derived growth factor A subunit precursor (PDGFA; PDGF-1)	X06374	P04085
PRB-binding protein E2F1; retinoblastoma-binding protein 3 (RBBP3); retinoblastoma-associated protein 1 (RBAP1); PBR3	M96577	Q01094; Q92768; Q13143
prostaglandin G/H synthase 1		P23219
protein-tyrosine phosphatase 1C (PTP1C); hematopoietic cell protein-tyrosine phosphatase; SH-PTP1	X62055	P29350
prothrombin precursor; coagulation factor II	V00595	P00734
proto-oncogene tyrosine-protein kinase lck; p56-lck; lymphocyte-specific protein tyrosine kinase (LSK); T-cell-specific protein-tyrosine kinase	U07236	P06239
P-selectin precursor (SELP); granule membrane protein 140 (GMP140); PADGEM; CD62P antigen; leukocyte-endothelial cell adhesion molecule 3 (LECAM3)	M25322	P16109
purine-rich single-stranded DNA-binding protein alpha (PURA)	M96684	Q00577
rab geranylgeranyl transferase alpha subunit	Y08200	Q92696
rab geranylgeranyl transferase beta subunit	Y08201	P53611; Q92697
RalB GTP-binding protein	M35416	P11234
ras-related C3 botulinum toxin substrate 2; p21-rac2; small G protein	M64595; M29871	P15153

ras-related protein RAB5A	M28215	P20339
related to receptor tyrosine kinase (RYK)	S59184	P34925
replication protein A 70-kDa subunit (RPA70; REPA1; RF-A); single-stranded DNA-binding protein	M63488	P27694
rho GDP dissociation inhibitor 2 (RHO GDI2; RHO-GDI beta); LY-GDI; ARHGDIB; GDID4	L20688	P52566
rho-GAP hematopoietic protein C1 (RGC1); KIAA0131	X78817	P98171
rho-related GTP-binding protein (RHOG); ARHG	X61587	P35238
ribonuclease 6 precursor	U85625	O00584
ribosomal protein S6 kinase II alpha 1 (S6KII-alpha 1); ribosomal S6 kinase 1 (RSK1)	L07597	Q15418
S100 calcium-binding protein A1; S-100 protein alpha chain	X58079	P23297
SCGF-beta	D86586	BAA21499
SEC7 homolog B2-1	M85169	Q15438
selectin P ligand	U02297	Q14242; Q12775
semaphorin; CD100	U60800	Q92854
serum response factor (SRF)	J03161	P11831
SH3-binding protein 2	AF000936	P78314
signaling inositol polyphosphate 5 phosphatase; SIP-110	U50040	Q13544
sonic hedgehog (SHH)	L38518	Q15465
specific 116-kDa vacuolar proton pump subunit	U45285	Q13488

sterold 5-alpha reductase 1 (SRD5A1); 3-oxo-5-alpha steroid 4 dehydrogenase 1	M32313; M68886	P18405
stromal cell derived factor 1 receptor (SDF1 receptor); fusin; CXCR4; leukocyte-derived seven transmembrane domain receptor (LESTR); LCR1	D10924	P30991
superoxide dismutase 2	M36693	P04179
T-cell surface glycoprotein CD3 epsilon subunit precursor; T-cell surface antigen T3/leu-4 epsilon subunit (T3E)	X03884	P07766
tenascin precursor (TN); hexabrachion (HXB); cytotactin; neuronectin; GMEM; miotendinous antigen; glioma-associated extracellular matrix antigen	X78565; M55618	P24821; Q15567; Q14583
thrombospondin 1 precursor (THBS1; TSP1)	X14787	P07996
thymidine phosphorylase precursor (TDRPase); platelet- derived endothelial cell growth factor (PDECGF); gliostatin	M63193	P19971; Q13390
TNF-related apoptosis inducing ligand (TRAIL); APO-2 ligand (APO2L)	U57059	P50591
TRAIL receptor 3; decoy receptor 1 (DCR1)	AF016267	O14755
transcription factor Spi-B	X66079	Q01892
transcriptional regulator interferon-stimulated gene factor 3 gamma subunit (ISGF3G); interferon-alpha (IFN-alpha) responsive transcription factor subunit	M87503	Q00978
transforming growth factor-beta 3 (TGF-beta3)	J03241	P10600
tuberin; tuberous sclerosis 2 protein (TSC2)	X75621	P49815

type I cytoskeletal 18 keratin; cytokeratin 18 (K18)	M26326	P05783
type II cytoskeletal 6 keratin: cytokeratin 6A (CK6A); K6A keratin (KRT6A) + CK6B; KRT6B + CK6C; KRT6C + CK6D; KRT6D + CK6E; KRT6E + CK6F; KRT6F	J00269 + L42592+ L42601 + L42610 + L42611 + L42612	P02538
tyrosine-protein kinase lyn	M16038	P07948
tyrosine-protein kinase receptor UFO precursor; axl oncogene	M76125	P30530
vascular endothelial growth factor B precursor (VEGFB) + VEGF-related factor isoform VRF186	U48801; U43369	P49765
vav oncogene	X16316	P15498
v-erbA related protein (EAR2)	X12794	P10588
versican core protein precursor; large fibroblast proteoglycan; chondroitin sulfate proteoglycan core protein 2; glial hyaluronate-binding protein (GHAP)	U16306; X15998; U26555; D32039	P13611
vitamin K-dependent protein S	Y00692	P07225

For example, it was found that 17 of the genes differentially expressed in human neointima encode transcriptional regulators. mRNA levels for 14 transcription factors were induced in neointima and 3 showed a decreased expression (Fig. 15). Some transcription factors of the former group have previously been related to proliferation and apoptosis of SMCs, such as HMG-1, E2F1, IRF-1, Fli-1, and with pro-inflammatory signaling in human neointima, such as IRF-1, IRF-7 and RelB. The following transcription factors were upregulated: E2F1, estrogen-related receptor alpha, ets domain protein elk-3, fli-1 oncogene, HMG-1, interferon regulatory factor 1, interferon regulatory factor 7, ISGF3-gamma, nuclear receptor-related 1, RELB, transcription factor Spi-B, vav oncogene, v-erbA related protein, vitamin D3 receptor; whereas the following were downregulated: homeobox protein HOXB7, early growth response protein 1, serum response factor.

Striking changes seem to take place in the expression of transcription factors of the Ets family. Whereas Spi-B, the fli-oncogene, and the Ets-repressor Elk-3 were induced in neointima, the Ets transcription factor Egr-1 was repressed (Figs. 14 and 15).

Furthermore, a number of genes involved in controlling or mediating proliferative responses were differentially expressed between neointima and control groups. The platelet-derived growth factor (PDGF)-A and angiotensinogen genes, whose products act on SMCs as mitogens, were exclusively expressed in neointima (Fig. 14). Angiotensin is known to be upregulated by insulin and to induce the expression of PDGF-A in SMCs. As a sign of ongoing proliferation, several genes known to be expressed with the G1/S transition of the cell cycle were found to be upregulated in neointima. Those include transcription factor E2F1, 70-kDa replication protein A, oncogene product Pim-1 and geranylgeranyl transferase. In addition, upregulation of the cell-cycle regulated histone H4, which is expressed in the G/S1 and S-phase of the cell cycle indicating ongoing proliferation in human neointima, was observed.
Reprogramming of cell growth in neointima evidently led to induction of several genes in neointima encoding proteins with functions in different signal transduction pathways, including the cell surface receptors EDG-1, EDG-4, insulin receptor and P2X

purinoceptor 5, and other signaling proteins like the ribosomal protein S6 kinase II alpha 1, farnesyltransferase, phospholipase C beta 2, growth factor receptor-bound protein 2, and the small G proteins CDC42, RhoG, p21-Rac2 and RalB. The enzyme farnesyltransferase catalyzes the essential post-translational lipidation of Ras and several other signal transducing G proteins. G proteins, like p21-Rac2, CDC42 and RhoG play pivotal roles in signal transduction pathways leading to cell migration and cell proliferation. Likewise, agonist-stimulated 1,4,5-triphosphate (IP3) production by phospholipase C beta 2 in smooth muscle requires G protein activation and activated Rac and Cdc42 associate with PI 3 kinase that plays an important role in the activation of the p70 S6 kinase. The p70 S6 kinase (p70S6K) is an important regulator of cell cycle progression to enter G1 phase and to proceed to S phase in response to growth factors and mitogens. It is involved in multiple growth factor related signal transduction pathways that are known to play pivotal roles in neointima formation, like angiotensin, endothelin and PDGF. In line with upregulation of p70 S6 kinase, significant upregulation of the FK506-binding protein (FKBP) 12 at mRNA (Fig. 14) and protein level in neointima was found.

It was observed that a number of genes encoding inhibitors of cell cycle progression were expressed in quiescent media but significantly downregulated in neointima (Fig. 14). Those included CIP1, p16-INK4, metallothionein, TGF-beta3, mammary-derived growth inhibitor, FrzB and the Gadd45 beta and gamma subunits.

Additionally, upregulation of genes in human neointima encoding proteins with pro-apoptotic function, like caspase-1, DAP-1 and APO-2 ligand, as well as upregulation of genes encoding proteins with anti-apoptotic function, like BAG-1, BCL-2-related protein A1 and the Trail receptor 3 (Fig. 14) was found.

Finally, the human neointima transcriptome showed upregulation of 32 genes related to IFN-γ signaling (Fig. 16). The IFN-γ receptor alpha was expressed in neointima, proliferating CASMCs and – to a lesser degree – in blood cells; whereas the IFN-γ receptor beta was mainly expressed in neointima specimen. Likewise, an upregulation of Pyk2 was observed.

Upregulation of the IFN-γ regulated genes for caspase-1, caspase-8 and DAP-1 was found in human neointima. However, mRNAs for the anti-apoptotic proteins BAG-1, Pim-1 (both regulated by IFN-γ) and BCL-2-related protein A1 were also upregulated in neointima versus control (Fig. 14).

Numerous genes with functions in inflammatory responses were found activated in human neointima. Pro-inflammatory gene patterns came from infiltrating inflammatory cells such as macrophages and T lymphocytes (e.g., CD11b, CD3) (Fig. 14C) or from neointimal SMCs (e.g., prostaglandin G/H synthase 1, phospholipase A2, heat shock protein 70, C5a anaphylatoxin receptor, IFN-γ receptor) (Fig. 14A and B).
The selective expression of CD40 in neointima deserves attention (Fig. 14A). CD40 is a member of the TNF receptor family that was initially described on the surface of B cells.

The following cytoskeletal, extracellular matrix and cell adhesion changes in neointima were observed:
An upregulation of connexin43 and of cytokeratin-18 in neointima as is seen in proliferating CASMC (Fig. 14B, upper panel), whereas the expression of desmin was strongly reduced in neointima (Fig. 14D, upper panel).
Whereas the transcription of different collagen subtypes and tenascin were reduced in neointima (Fig. 14D, upper panel), expression of thrombospondin-1 and versican were upregulated (Fig. 14B, upper panel).
A number of genes encoding adhesion molecules, including P-selectin, ICAM2 and cadherin16, were found highly expressed in neointima but not in SMCs, blood cells or control vessels (Fig. 14A, upper panel). A number of other adhesion molecules were similarly expressed in neointima, cultured SMCs (Fig. 14B) and blood cells (Fig. 14C). Neointima appears to downregulate expression of certain adhesion molecules that are normally expressed in media/intima of arteries, such as integrins α7B, α3 or MUC18.

Example VII: Upregulated genes of the IFN-γ signaling pathway

As shown herein above, the expression of 2,435 genes of known function in atherectomy specimen of 10 patients with in-stent restenosis, blood cells of 10 patients,

normal coronary artery specimen of 11 donors, and cultured human coronary artery smooth muscle cells was investigated and 224 genes that were differentially expressed with high statistical significance ($p<0.03$) between neointima and control tissue were identified. In particular, 32 upregulated genes that are related to interferon-γ signaling were identified in neointima.

The IFN-γ receptor alpha was expressed in neointima, proliferating CASMCs and –to a lesser degree– in blood cells; whereas the IFN-γ receptor beta was mainly expressed in neointima specimen.

IFN-γ signals via a high-affinity receptor containing an α- and β-receptor chain. Interstingly, TH1 cells use receptor modification to achieve an IFN-γ-resistant state (Pernis, Science 269 (1995), 245-247). The subtype-specific difference in the activation of the IFN-γ signaling pathway of type 1 and type 2 T helper cells is due to a lack of IFN-γ receptor ß in type 1 T cells. Therefore, the here presented data would argue that a high affinity IFN-γ receptor containing both chains is mainly expressed in smooth muscle cells of the neointima.

Consistent with an activation of IFN-γ signaling, upregulation of two transcription factors in neointima that are essential for IFN signalling were found: IRF-1 and ISGF3γ (p48). These transcription factors are known to be transcriptionally upregulated by IFN-γ (Der, Proc. Natl. Acad. Sci. 95 (1998), 15623-15628), and both are key players in IFN-γ signalling (Matsumoto, Biol. Chem. 380 (1999), 699-703; Kimuar, Genes Cells 1 (1996), 115-124; Kirchhoff, Nucleic Acids Res. 21 (1993), 2881-2889; Kano, Biochem. Biophys. Res. Commun. 257 (1999), 672-677). Likewise, upregulation of the tyrosine kinase Pyk2 was observed, which has been shown to play a role in the signal transduction by angiotensin in SMCs (Sabri, Circ. Res. 83 (1998), 841-851). Pyk2 is selectively activated by IFN-γ and inhibition of Pyk2 in NIH 3T3 cells results in a strong inhibition of the IFN-γ-induced activation of MAPK and STAT1 (Takaoka, EMBO J. 18 (1999), 2480-2488.

A key event in IFN-γ-induced growth inhibition and apoptosis is the induction of caspases (Dai, Blood 93 (1999), 3309-3316). It has been shown that IRF-1 induces

expression of caspase-1 leading to apoptosis in vascular SMCs (Horiuchi, Hypertension 33 (1999), 162-166), and that apoptotic SMCs and macrophages colocalize with caspase-1 in atherosclerosis (Geng, Am. J. Pathol. 147 (1995), 251-266). In this studies, upregulation of the IFN-γ–regulated genes for caspase-1, caspase–8 and DAP-1 in human neointima was found. However, mRNAs for the the anti-apoptotic proteins BAG-1, Pim-1 (both regulated by IFN-γ) and BCL-2-related protein A1 were also upregulated in neointima versus control (Fig. 16), supporting the notion that proliferation and apoptosis occur simultaneously in human neointima with a preponderance of proliferation.

Coordinated regulation of genes whose products act at different steps in the neointima process was a recurring theme of our gene expression analysis. Regarding the IFN-γ pathway, not only the genes for the complete receptor, the main transcription factors, components of the signal transduction pathway (Dap-1, BAG-1, Pim-1, IFN-γ-inducible protein, IFN-inducible protein 9-27) were induced but also several target genes of the IFN-γ pathway, like CD40, CD13 and thrombospondin-1 (Fig. 16).

The IFN-γ-regulated gene cluster was expressed in the neointima specimen but some of the relevant genes, like IRF-1, were also expressed in blood samples. To identify the cell type that predominantly contributed to the IFN-γ regulated pattern, frozen sections of neointima specimen from coronary in-stent restenosis (n=3) and from restenosis of peripheral arteries (n=6) were stained with antibodies specific for IRF-1. This protein was chosen because it is an essential component of the IFN-γ signal transduction pathway (Kimura, loc. cit.) and was expressed coordinately with the other genes in the cluster (Fig. 16). Immunohistochemical analysis showed strong nuclear and cytoplasmic staining of IRF-1 in neointimal SMCs of a carotid restenosis (Fig. 17) and of coronary in-stent restenosis (Fig. 18), as identified by their spindle-shaped nuclei and by staining with the smooth muscle cell marker alpha-actin (Fig. 18). The nuclear staining of IRF-1 in in-stent restenosis (Fig. 18) indicated that the IRF-1 transcription factor is also activated. SMCs in control media of carotid arteries did not show IRF-1 staining (Fig. 17). CD3-positive cells were much less abundant in the specimen (Fig. 18) than SMCs (Fig. 18), indicating that SMCs contributed mostly to the increased IRF-1 expression in human neointima.

The presence of IFN-γ in human atherosclerotic lesions is well established (Ross, N. Engl. J. Med. 340 (1999), 115-126) although its role remains unclear. Whereas IFN-γ inhibits proliferation and induces apoptosis in SMCs in vitro (Horiuchi, loc. cit.; Warner, J. Clin. Invest 83 (1989), 1174-1182), absence of IFN-γ reduces intima hyperplasia in mouse models of atheroma and transplant arteriosclerosis (Gupta, J. Clin. Invest 99 (1997), 2752-2761; Raisanen-Sokolowski, Am. J. Pathol. 152 (1998), 359-365). In line with this observation, it was shown that IFN-γ induces arteriosclerosis in absence of leukocytes in pig and human artery tissues by their insertion into the aorta of immunodeficient mice (Tellides, Nature 403 (2000), 207-211).

The role of infiltrating T lymphocytes in neointima of in-stent restenosis has not been examined yet. In this study it was shown that CD3-positive cells can be detected by immunobiochemists in 3 out of 4 neointima samples (see Fig. 18), and a CD3-specific hybridization signal on cDNA arrays with 7 out of 10 neointima specimen was obtained (Fig. 18). IFN-γ-related expression patterns were also observed in samples negative for CD3 as examined by either method, suggesting that the cytokine could act on neointima in a paracrine fashion over some distance with no need for massive T cell infiltration.

While T cells and the pro-inflammatory cytokine IFN-γ are known to play an important role in atherosclerosis (Ross, loc. cit.), their role in the development of neointima is largely unexplored. The here provided data suggest an important role of IFN-γ in the pathophysiology of neointimal hyperplasia.

Example VIII: Preparation of a surrogate cell line

A surrogate cell line for a pathologically modified cell and/or tissue may be prepared by the following steps:

a) **Definition of the transcriptome/gene expression pattern of the diseased tissue:**

Microscopic specimen of diseased tissue may be obtained by either atherectomy, debulking, biopsy, laser dissection of diseased tissue or macroscopic surgical dissection of diseased tissue. After acquisition, microscopic specimen are immediately frozen in liquid nitrogen and kept in liquid nitrogen until mRNA preparation is performed in order to preserve the in vivo status of the samples' transcriptomes.

The cells in such samples express a particular set of genes which is reflected by the presence of distinct mRNA molecules occuring at various concentrations. The entirety of mRNA molecules and their relative amounts in a given clinical sample is referred to as the transcriptome. The transcriptome of a diseased tissue is expected to be different from that of a healthy tissue. The differences relate to the up- or downregulated expression of genes involved in causing, maintaining or indicating the diseased state of the tissue. The analysis of the transcriptome is typically limited by the number of cDNA elements a particular array carries.

mRNA preparation and amplification is carried out according to the method of the invention and described herein above.

In particular, microscopic specimen of diseased tissue are quick-frozen and kept in liquid nitrogen until mRNA preparation and cDNA synthesis is performed as described herein above. Frozen tissue is ground in liquid nitrogen and the frozen powder dissolved in Lysis buffer according to the procedure of RNA preparation. The lysate is centrifuged for 5 min at 10, 000 g at 4° to remove cell debris. RNA

can be prepared as total RNA or as mRNA as described ein (Schena, Science 270 (1995), 467-470), in Current Protocols, in the Clontech manual for the Atlas cDNA Expression Arrays or as described in (Spirin, Invest. Ophtalmol. Vis. Sci. 40 (1999), 3108-3115), as described in (Chee, Science 274 (1996), 610-614; Alon, Proc. Natl. Acad. Sci. 96 (1999), 6745-6750; Fidanza, Nucleosides Nucleotides 18 (1999), 1293-1295; Mahadevappa, Nat. Biotechnol. 17 (1999), 1134-1136; Lipshutz, Nat. Genet. 21 (1999), 20-24) for the Affymetrix arrays or as described by Qiagen.

cDNA preparation and labeling can be performed as described by Clontech or Affymetrix in the user's manual for the arrays hybridization kits or as described in (Spirin, loc. cit.; Chee, loc. cit.; Alon, loc. cit.; Fidanza, loc. cit.; Mahadevappa, loc. cit.; Lipshutz, loc. cit.). Additionally, amplified cDNA can be used. Preparation of cDNA amplificates and labeling of amplificated cDNA can be performed as described herein above or by Spirin (loc. cit.).

Obtained, labeled cDNA can be employed in hybridization assays. Hybridization of labeled cDNA and data analysis can be performed under conditions as described in the user's manual from Clontech's AtlasTM cDNA Expression Arrays User Manual or in the manufacter's manual of Affymetrix or as described by (Spirin, loc. cit.; Chee, loc. cit.; Alon, loc. cit.; Fidanza, loc. cit.; Mahadevappa, loc. cit.; Lipshutz, loc. cit.).

b) Definition of the transcriptome/gene expression pattern of control tissue

To identify disease-specific gene expression patterns, the gene expression pattern of the diseased tissue can be compared to control material from healthy donors. In the case of atherectomy material this can be healthy media and intima of non-elastic, i.e., muscular arteries. In the case of heart muscle biopsies or kidney biopsies, healthy control tissue can be used that is collected in the course of the operation. Additionally, gene expression pattern of cells of neighbouring unaffected tissue or of infiltrating cells, like blood, cells can be analyzed. Based

upon the celluar characterization of a tissue by immunohistochemical analysis using antibodies to cell marker proteins, transcriptome can be determined from cultured human cell lines of the same type. (Example: arteries stain positive for smooth muscle cells and endothelial cells; consequently transcriptomes are obtained from cultured human smooth muscle and endothelial cells).

mRNA preparation and amplification can be carried out as described herein above and in accordance with the method of the present invention. Obtained (labeled) cDNA may be employed in hybridization assays as described herein above.

c) Determination of a relevant set of disease specific genes

To determine disease-specific gene expression patterns first the gene expression pattern of the diseased tissue should be compared to the gene expression pattern of healthy control tissue. For comparison, the mean expression value of at a sufficient number of diseased specimen (e.g., 10) and the same number of control specimen should be compared. Genes with an expression ratio >2.5-fold between the the two groups should be analyzed for their relative expression in one group: there should be >5/10 positive in one group, if there are 0/10 in the other or at least 7/10 in one group if there are maximally 3/10 positive in the other group. Additionally, these data should be analysed statistically to define genes with an $p<0.05$ with e.g. the Wilcoxon test as described in the manual of SPSS 8.0.

Genes selected based upon their significant over- or underexpression by a factor of 2.5 are refered to as aberrantly regulated in the diseased tissue, or as diseases-related genes. Disease-related genes genes are then grouped by the functions of encoded proteins. e.g. genes encoding proteins of the signalling pathway, cytokines, chemokines, hormones, their receptors, proteins specific or infiltrating cells, or proteins involved in extracellular matrix, cell adhesion, migration, cell division, cell cycle arrest. Likewise genes of unknown function, as

available thorugh public EST data bases, can be identified as being disease-related.

d) Screen for a cell line with a transcriptome most closely resembling that of diseased tissue

Drugs that can potentially regulate the expression of diseased genes can be discovered by screening large libraries of chemicals or biologics. In order to identify such drugs, a screening cell line must be available that faithfully reflects the transcriptome of the diseased tissue and is avaiabale in large quantaties for the performance of a comprehensive drug screen. Moreover information is needed of how the drug candidate should alter the transcriptome of the cell line that has characteristics of the transcriptome of the diseased tissue. This information is obtained from the transcriptome of the healthy control tissue. The drug should be able to re-estsblish features of a "healthy" transcriptome.

A human cell line, which is most similiar to the cellular origin of the diseased tissue, e.g, coronary artery smooth muscle cells for atherectomy, HepG2 cells for liver diseases, renal cells for kidney diseases or cardiomyoblasts for heart muscle disease should be used. Cells should be grown under standard conditions as described in the manufacter's manual like the ones from ATCC.

Transcriptome analysis/gene expression pattern analysis can be performed as described for the diseased and the control tissue and gene expression pattern should be compared to the gene expression pattern of the diseased and the healthy tissue. For generating a surrogate screening cell line, the cell line which shows a transcriptome most similar to the diseased transcriptome should be selected.

e) **Adaptation of a cell line to mimick diseased transcriptome/gene expression pattern**

In order to generate a surrogate screening cell line for the diseased tissue, it may be necessary to adapt the transcriptome of the selected cell line to the transcriptome of the diseased tissue. This can on the one hand be achieved by incubation of the cell line with compounds such as cytokines or hormones, that had been shown to play an important role in the gene expression pattern of the diseased tissue. Likewise such compounds can be identified by transcriptome analysis of diseased tissue as exemplified with neoinitima where evidence for a role of interferon-gamma was obtained. Instead of addition of compounds with relevance for the disease, the screening cell line can be conditioned by co-culture with other cell types relevant for the pathophysiology of the disease. Such cells can for instance be inflammatory cells, like macrophages or T cells, that migrate into the diseased tissue and by released factors or cell-cellcontact contribute to the disease-specific gene expression pattern. In each case, transcriptome analysis of the surrogate line must identify the optimal addition to generate a disease-specific expression pattern.

Compounds that can be used for adapting the transcriptome of a surogate cell line to the diseased state comprise cytokines, growth factors, small molecule compounds (drugs), or peptides and peptidomimetics. Cell lines that can be used for such an adaptation comprise human monocytic cell lines, like U937, THP-1 or Monomac-6, or human T-cell lines like Jurkat.

The co-culture/treatment conditions leading in the surrogate cell line to a state closest to the diseased transcriptome are selected for drug screening.

In the following, a specific example should illustrate the preparation of a surrogate. In particular, a surrogate cell (line) for restenotic tissue is prepared by the following steps:

a) **Aquisition of in-stent restenotic tissue**

Patients

The in-stent restenosis study group consisted of 13 patients who underwent separate atherectomy procedures by X-sizer within the renarrowed stent between 4-23 month after primary stent implantation. All patients gave informed consent to the procedure and received 15,000 units heparin before the intervention followed by intravenous heparin infusion, 1,000 units/h for the first 12 h after sheat removal as standard therapy. All patients received aspirin, 500 mg intravenously, before catherisation, and postinterventional antithrombotic therapy consisted of ticlopidine (250 mg bds) and aspirin (100mg bds) throughout the study.

Sample Preparation

Atherectomy specimen were immediately frozen in liquid nitrogen after debulking of the lesion, and kept in liquid nitrogen until mRNA preparation was performed as described. For histology and immunhistochemistry of the in-stent restenotic tissue from coronary arteries (n=3), the samples were fixed in 4% paraformaldehyd and embedded in paraffin as described.

Morphological characterization of restenotic tissue

Immunohistochemistry for cell typing was performed on paraffin-embedded sections of three neointima specimen from coronary in-stent restenosis and, for detection of FKBP12, on frozen sections of four neointima specimen from carotid restenosis. Three µm serial sections were mounted onto DAKO ChemMateTM Capillary Gap Microscope slides (100 µm) baked at 65°C overnight, deparaffinized and dehydrated according to standard protocols. For antigen retrieval, specimens were boiled 4 min in a pressure cooker in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase was blocked by 1% H2O2/methanol for 15 minutes. Unspecific binding of the primary antibody was reduced by preincubation of the slides with 4% dried skim milk in Antibody Diluent (DAKO, Denmark). Immunostaining was performed by the streptavidin-peroxidase technique using the ChemMate Detection Kit HRP/Red Rabbit/Mouse (DAKO,

Denmark) according to the manufacturer's description. The procedures were carried out in a DAKO TechMateTM 500 Plus automated staining system. Primary antibodies against smooth muscle actin (M0635, DAKO, Denmark; 1:300), CD3 (A0452, DAKO, Denmark; 1:80), MAC387 (E026, Camon, Germany; 1:20) and FKBP12 (SA-218, Biomol, Germany, 1:20) were diluted in Antibody Diluent and incubated for 1 h at room temperature. After nuclear counterstaining with hematoxylin, the slides were dehydrated and coverslipped with Pertex (Medite, Germany).

The Cellular Composition of Debulked In-stent Restenotic Material

Representative samples obtained from x-sizer treatment of a neointimal hyperplasia were analyzed by immunhistochemistry in order to determine its cellular composition. Figure 7A shows an E.-van-Giesson staining of a section cut from a small sample of debulked restenotic material. With this staining procedure, collagen fibers stain red, fibrin stains yellow and cytoplasm of smooth muscle cells stains dark-yellow-brown. The majority of the volume of debulked material was composed of loose extracellular matrix-like collagen fibers stained in light red. Yellow fibrin staining was barely detectable. Cells with spindle-shaped nuclei and a yellow/brown-stained cytoplasm were frequent. Their identity as smooth muscle cells and their high abundance in restenotic material was supported by immunostaining with an antibody against smooth muscle α-actin (Fig. 7B). There, the staining pattern of a section from an entire specimen as used for gene expression analysis is shown. As described below, such samples also gave raise to a strong smooth muscle-specific α-actin mRNA signal (see Fig. 8). These results support findings from previous studies (Kearney, Circulation 95 (1997), 1998-2002; Komatsu, Circulation 98 (1998), 224-233; Strauss, J. Am. Coll. Cardiol. 20 (1992), 1465-1473)demonstrating that the main cell type found in neointima is derived from smooth muscle cells. As described in the literature(Kearney, loc. cit.; Komatsu, loc. cit.; Strauss, loc. cit.) mononuclear infiltrates could also be identified in some areas of debulked restenotic tissue specimen. These infiltrates consisted mainly of macrophages and to a lesser

degree of t-lymphocytes. No b-lymphocytes were detectable in the restenotic tissue by using an antibody against CD20 for immunhistochemical staining.

b) Transcriptome analysis of restenotic material

Transcriptome analysis of neointima was performed using the method of mRNA amplification as described herein above.

mRNA Preparation

Microscopic specimen diseased tissue were quick-frozen and kept in liquid nitrogen until mRNA preparation and cDNA synthesis was performed. Frozen tissue is ground in liquid nitrogen and the frozen powder dissolved in Lysis/Binding buffer (100mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol (DTT)) and homogenized until complete lysis is obtained. The lysate is centrifuged for 5 min at 10, 000 g at 4° to remove cell debris. mRNA is prepared using the Dynbeads® mRNA Direct KitTM (Dynal, Germany) following the manufacture's recommendation. Briefly, lysate was added to 50 µL of pre-washed Dynabeads Oligo (dT)25 per sample and mRNA was annealed by rotating on a mixer for 30 min at 4°C. Supernatant was removed and Dynabeads Oligo (dT)25/mRNA complex was washed twice with washing buffer containing Igepal (50mM Tris-HCl, pH 8.0, 75 mM KCl, 10 mM DTT, 025% Igepal), and once with washing buffer containing Tween-20 (50mM Tris-HCl, pH 8.0, 75 mM KCl, 10 mM DTT, 0.5% Tween-20).

Preparation of Amplified cDNA

cDNA is amplified by PCR using the procedure of Klein et al. (C. Klein et al.). First-strand cDNA synthesis is performed as solid-phase cDNA synthesis. Random priming with hexanucleotide primers is used for reverse transcription reaction. mRNAs are each reversely transcribed in a 20 µL reaction volume containing 1x First Strand Buffer (Gibco), 0.01 M DTT (Gibco), 0.25 % Igepal, 50 µM CFL5c-Primer [5'-(CCC)5 GTC TAG A (NNN)2-3'], 0.5 mM dNTPs each (MBI Fermentas) and 200 U Superscript II (Gibco), and incubate at 44°C for 45 min. A

subsequent tailing reaction is performed in a reaction volume of 10 µL containing 4 mM MgCl2, 0.1 mM DTT, 0.2 mM dGTP, 10 mM KH2PO4 and 10 U of terminal deoxynucleotide transferase (MBI Fermentas). The mixture is incubated for 24 min at 37°C.

cDNA is amplified by PCR in a reaction volume of 50 µL containing 1 x buffer 1 (ExpandTM Long Template PCR Kit, Boehringer Mannheim), 3% deionized formamide, 120 µM CP2-Primer [5'-TCA GAA TTC ATG (CCC)5-3'], 350 µM dNTP and 4.5 U DNA-Polymerase-Mix (ExpandTM Long Template PCR Kit, Roche Diagnostics, Mannhein). PCR reaction is performed for 20 cycles with the following cycle parameters: 94°C for 15 sec, 65°C for 0:30 min, 68°C for 2 min; for another 20 cycles with: 94°C for 15 sec, 65°C for 30 sec, 68°C for 2:30 + 0:10/cycle min; 68°C 7 min; 4°C forever.

Expression of Specific Genes In Microscopic Human Tissue Samples

In order to optimally preserve the in situ mRNA levels, restenotic and control specimen were immediately frozen after harvest in liquid nitrogen and carefully lyzed as described in Materials and Methods. After PCR amplification of the synthesized cDNA the amount of the amplified cDNA was measured by a dot blot assay and found to be between 200-300 ng/µl. The quality of every amplified cDNA sample was tested by gene-specific PCR using primers detecting cDNAs for ß-actin, smooth muscle cell α-actin and the ubiquitous elongation factor EF-1α. Figure 8 shows a representative result with material from patient B and control media from donor b. In both specimen, PCR signals of the correct size from house-keeping genes ß-actin and EF-1α were detectable in equivalent amounts (compare lanes 1 and 2 with lanes 4 and 5). Additionally, α-actin signals as marker for smooth muscle cells was obatined from each specimen (lanes 3 and 6). These results show that mRNA prepraration, cDNA synthesis and PCR amplification of cDNA is feasible with microscopic human restenosis samples.

Dig-dUTP Labeling of cDNA Probes

25 ng of each cDNA is labeled with Digoxigenin-11-dUTP (Dig-dUTP) (Roche Diagnostics) during PCR. PCR is performed in a 50 µL reaction with 1x Puffer 1,

120 µM CP2 primer, 3% deionized formamide, 300 µM dTTP, 350 µM dATP, 350 µM dGTP, 350 µM dCTP, 50 µM Dig-dUTP, 4.5 U DNA-Polymerase-Mix. Cycle parameters are: one cycle: 94°C for 2 min; 15 cycles: 94°C for 15 sec, 63°C for 15 sec, 68°C for 2 min; 10 cycles: 94°C for 15 sec, 68°C for 3 min + 5 sec/cycle; one cycle: 68°C, 7 min, 4°C forever.

Hybridization of Clontech cDNA Arrays with dUTP-labeled cDNA Probes

cDNA arrays are prehybridized in DigEASYHyb solution (Roche Diagnostics) containing 50mg/L DNAseI (Roche Diagnostics) digested genomic E. coli DNA, 50mg/L pBluescript plasmid DNA and 15 mg/L herring sperm DNA (Life Technologies) for 12h at 44°C to reduce background by blocking non-specific nucleic acid-binding sites on the membrane. Hybridization solution is hybridized to commercially available cDNA arrays with selected genes relevant for cancer, cardiovascular and stress response (Clontech). Each cDNA template is denatured and added to the prehybridization solution at a concentration of 5 µg/ml Dig-dUTP-labeled cDNA. Hybridization was performed for 48 hours at 44°C.

Blots are subsequently rinsed once in 2x SSC/0.1% SDS and once in 1x SSC/0.1% SDS at 68°C followed by washing for 15 min once in 0.5x SSC/0.1% SDS and twice for 30min in 0.1x SSC/0.1%SDS at 68°C. For detection of Dig-labeled cDNA hybridized to the array, the Dig Luminescent Detection Kit (Boehringer, Mannheim) was used as described in the user manual. For detection of the chemiluminescence signal, arrays are exposed to chemiluminescence films for 30 min at room temperature. Quantification of array data was performed by scanning of the films and analysis with array vision software (Imaging Research Inc., St. Catharines, Canada). Background was subtracted and signals were normalized to the nine housekeeping genes present on each filter, whereby the average of the housekeeping gene expression signals was set to 1 and the background set to 0.

Each labeled probe was hybridized to three different commercial cDNA arrays which allowed for the expression analysis of a total of 2,435 known genes. Figure 9 shows a representative hybridization pattern obtained with one array using

probes prepared from restenotic tissue of patient B (panel A) and media of donor b (panel B). Consistent with the gene-specific analysis shown in Figure 8, comparable hybridization signals were obtained with the positive control of human genomic cDNA spotted on the right and bottom lanes of the array and with cDNA spots of various housekeeping genes (see for instance, spots D). If a biological specimen was omitted from cDNA synthesis and PCR amplification reactions almost no hybridization signals were obtained (Fig. 9, panel C), showing that hybridization signals were almost exclusively derived from added samples and not from DNA contaminations in reagents or materials used.

Data Analysis

Quantification of array data was performed by scanning of the films and analysis with array vision software (Imaging Research Inc., St. Catharines, Canada). Background was subtracted and signals were normalized to the nine housekeeping genes present on each filter, whereby the average of the housekeeping gene expression signals was set to 1 and the background set to 0. For the logarithmic presentation shown in Figure 13A and 13B, values were multiplied by 1000. A mean value >0,05 in the average of all samples in one group was regarded as a positive signal. Differences in the mean expression level by a factor >2.5-fold between the study and the control group were further statistically analyzed.

c) **Choice of control tissue**

As the main cellular component of neointima consists of smooth muscle cells, media and media/intima were taken of healthy coronary arteries or as coronary arteries belong to the non-elastic but muscular arteries muscular arteries as control tissue.

The control group consisted of 6 specimen from coronary arteries from three different patients who underwent heart transplantation. Additionally, 5 specimen of muscular arteries of the gastrointestinal tract from five different patients were taken as control because coronary arteries belong histologically to muscular arteries. The control specimen were immediately frozen in liquid nitrogen. Prior to mRNA preparation, media and intima of the control arteries were prepared and examined for atherosclerotic changes by immunhistochemistry. If there were no atherosclerotic changes of the vessel morphology, the specimen (approx. 1x1 mm) were used as healthy control samples and mRNA and cDNA preparation and transcriptome analysis was performed as described above for neointimal tissue.

d) **Definition of the neointima-specific gene expression profile**

A total of 1,186 genes (48.7 %) out of 2,435 yielded detectable hybridization signals on cDNA arrays with neointima and control samples over a 20-fold range of expression level (Fig. 13A) Thereof 352 genes (14.5 %) appeared to be differentially expressed by a factor >2.5-fold between restenotic and control samples. However, expression levels considerably varied among individual samples (see, e.g., Fig. 9). A statistical analysis was therefore employed in order to identify those genes that are differentially expressed between study and control groups with high significance (see herein above). This way, 224 genes (9.6%) were identified that were differentially expressed by a factor of at least 2.5-fold between the restenosis study group and the control group with a significance in the Wilcoxon test of $p<0.03$. 167 (75%) genes thereof were found

overexpressed and 56 genes (25%) underexpressed in the restenosis study group compared to the control group (Fig. 13B).

e) Choice of surrogate cell line

Human neointima consists of a heterogenous cell population. It was therefore attempted to relate the differential, statistically relevant gene expression patterns found with neointima to patterns eventually contributed by peripheral blood cells of the patients and cultured human CASMCs, i.e., cells that are most frequently encountered in restenotic tissue(Komatsu, loc. cit.). With respect to neointima expression, the 224 aberrantly regulated genes fell into four subgroups (Fig. 14). Group I lists 62 genes that were overexpressed in neointima and not highly or detectably expressed in control vessels, CASMCs or blood cells (Fig. 14A). In group II, 43 genes are listed that are similarly expressed in neointima and CASMCs, suggesting that this gene cluster in neointima was contributed by proliferating SMCs (Fig. 5B). In group III, 62 genes are listed that are similarly expressed in neointima and blood cells, suggesting that this gene cluster was contributed to that of neointima by infiltrated blood cells (Fig. 14C). This notion is supported by the expression in group III of the largest number of genes related to inflammation in all four groups. Lastly, in group IV, 56 genes are listed that are downregulated in neointima compared to the control group (Fig. 14D).

Upregulation of γ-IFN-related Genes in Neointima

A surprising feature of the human neointima transcriptome was the apparently coordinate upregulation of 32 genes related to IFN-γ signaling (Fig. 16). The IFN-γ receptor alpha was expressed in neointima, proliferating CASMCs and -to a lesser degree– in blood cells; whereas the IFN-γ receptor beta was mainly expressed in neointima specimen. Consistent with an activation of IFN-γ signaling, upregulation of two transcription factors in neointima was found that are essential for IFN signalling: IRF-1 and ISGF3γ (p48) (Figs. 14, 15, 16). These transcription factors are known to be transcriptionally upregulated by IFN-γ, and

both are key players in IFN-γ signalling. Likewise, upregulation of the tyrosine kinase was observed Pyk2 (Fig. 16), which has been shown to play a role in the signal transduction by angiotensin in SMCs (Sabri, Circ. Res. 83 (1998), 841-851). Pyk2 is selectively activated by IFN-γ and inhibition of Pyk2 in NIH 3T3 cells results in a strong inhibition of the IFN-γ-induced activation of MAPK and STAT1.

A key event in IFN-γ-induced growth inhibition and apoptosis is the induction of caspases(Dai, Blood 93 (1999), 3309-3316). In the here presented analysis on upregulation of the IFN-γ–regulated genes for caspase-1, caspase–8 and DAP-1 in human neointima. However, mRNAs for the the anti-apoptotic proteins BAG-1, Pim-1 (both regulated by IFN-γ) and BCL-2-related protein A1 were also upregulated in neointima versus control (Fig. 16), supporting the notion that proliferation and apoptosis occur simultaneously in human neointima with a preponderance of proliferation.

Coordinated regulation of genes whose products act at different steps in the neointima process was a recurring theme of our gene expression analysis. Regarding the IFN-γ pathway, not only the genes for the complete receptor, the main transcription factors, components of the signal transduction pathway (Dap-1, BAG-1, Pim-1, IFN-γ-inducible protein, IFN-inducible protein 9-27) were induced but also several target genes of the IFN-γ pathway, like CD40, CD13 and thrombospondin-1 (Fig. 16).

The IFN-γ-regulated gene cluster was expressed in the neointima specimen but some of the relevant genes, like IRF-1, were also expressed in blood samples. To identify the cell type that predominantly contributed to the IFN–γ regulated pattern, frozen sections of neointima specimen from coronary in-stent restenosis (n=3) and from restenosis of peripheral arteries (n=6) were stained with antibodies specific for IRF-1. This protein was chosen because it is an essential component of the IFN-γ signal transduction pathway (Kimura, Genes Cells 1 (1996), 115-124) and was expressed coordinately with the other genes in the cluster (Fig. 17). Immunohistochemical analysis showed strong nuclear and cytoplasmic staining of IRF-1 in neointimal SMCs of a carotid restenosis (Fig. 17B) and of coronary in-stent restenosis (Fig. 18C), as identified by their spindle-

shaped nuclei and by staining with the smooth muscle cell marker alpha-actin (Fig. 18B). The nuclear staining of IRF-1 in in-stent restenosis (Fig. 18C) indicated that the IRF-1 transcription factor is also activated. SMCs in control media of carotid arteries did not show IRF-1 staining (Fig. 17B). CD3-positive cells were much less abundant in the specimen (Fig. 18C) than SMCs (Fig. 18D), indicating that SMCs contributed mostly to the increased IRF-1 expression in human neointima.

Definition of culturing conditions in order to adapt transcriptome profile to that of restenotic tissue: IFN-γ

To adapt the transcriptional profile of cultured human coronary artery smooth muscle cells (CASMC) (Clonetics) to that of neointima, CASMC were stimulated with IFN-g and performed transcriptome analysis as described above. CASMC were cultured as described in the manufacter's manual in growth medium until 50% confluency was reached. Afterwards cells were stimulated with 1000U/ml IFN-γ (R&D, Germany) for 16 hours at 37°C. Cells were washed twice in PBS and RNA preparation, cDNA synthesis and amplification and transcriptome analysis was performed as described above.

As shown in Fig. 19 the neointima-specific IFN-γ gene expression pattern could be generated by incubation of CASMCs with 1000 U/ml IFN-γ.

Definition of the transcriptome/gene expression pattern of neointima after incubation with an IFN-γ antagonist

Microscopic specimen of in-stent restenotic tissue were incubated with an antagonist for IFN-γ for different times and transcriptome analysis was performed as described. Transcriptome of treated neointima was compared to the transcriptome of untreated neointima and healthy control tissue, to measured the therapeutic effect of IFN-γ antagonists.

Definition of the transcriptome/gene expression pattern of neointima after incubation with rapamycin

It has been shown in the literature, that rapamycin, a ligand of the intracellular protein FKBP12 inhibits migration and proliferation of smooth muscle cells and is able to reduce neointimal hyperplasia in a porcine model of restenosis. As significant upregulation of FKBP12 in the neointima specific transcriptome was found in order to evaluated the therapeutic effect of rapamycin.

As proliferating CASMC overexpress FKBP12 like neointima, this cell line can be employed as a potential surrogate cell line for neointima in respect to therapeutic effects of rapamycin. Therefore, in a first step, CASMC were incubated with 100ng/ml rapamycin (Sigma) for 24 hours and transcriptome analysis was performed in order to monitore the therapeutic effect. Afterwards, microscopic specimen of in-stent restenotic tissue are incubated with rapamycin and transcriptome analysis was performed as described herein above. Transcriptome/gene expression pattern of rapamycin treated CASMC was compared to the transcriptome of rapamycin-treated neointima to measured the effectiveness of CASMC as a surrogate cell line for neointima. Tumorsuppressor genes and proliferation-inhibiting genes have upregulated in said CASMCs; therefore said CASMCs can be considered as an true surrogate for neointima.

Example IX: Upregulated protein expression of Emmprin and transferrin receptor on tumor cells

Transcriptome analysis of single micrometastatic cells derived from patients with different tumor and disease stages revealed an upregulated expression of genes involved in cell cycle regulation, cytoskeleton organization, adhesion and proteolytic activity. Enhanced mRNA expression of Emmprin was found by array hybridization in 10 of 26 micrometastatic cells from bone marrow of breast and prostate cancer patients indicating an invasive phenotype of these cells. EMMPRIN (extracellular matrix metalloproteinase inducer, CD147) is a member of the immunoglobulin superfamily that is present on the surface of tumor cells and stimulates adjacent fibroblasts to produce matrix metalloproteinases (MMPs, Guo, J. Biol. Chem. 272 (1997), 24-27 and Sameshima, Cancer Lett. 157 (2000), 177-184 and Li, J. Cell Physiol. 186 (2001), 371-379). The results were controlled by gene specific PCR revealing a similar sensitivity compared to array hybridization. Using a different Emmprin-specific probe for array

hybridization, the Emmprin message was even detected in 16/26 (61%) samples. These results emphasize the sensitivity of the array design to detect the transcripts of a random primed single cell cDNA.

In order to correlate upregulation of Emmprin expression on tumor cells not only on mRNA but also on protein level, slides were prepared from bone marrow cells of cancer patients as described before (Pantel, Lancet 347 (1996), 649-653). Slides were blocked using 10% human AB serum in PBS for 20 min. From each sample one million bone marrow cells were screened for the presence of cytokeratin positive cells which is a marker for epithelial cells. A double staining procedure, employing the EMMPRIN specific antibody MEM 6/2 (Koch, Int. Immunol. 11 (1999), 777-86) and a biotin-conjugated A45B/B3 antibody reacting with several cytokeratin family members was performed. Antibody incubations were as follows: MEM 6/2, 45 min. 5 µg / ml; Z259 and APAAP complex according to the manufacturer's instructions (DAKO). Slides were washed 3 x 3 min. in PBS between all antibody incubations. Before the A45 B/B3-biotin F $(ab)_2$-fragment was added, an additional blocking step with 10% mouse serum in PBS was performed for 20 min. The A45 B/B3-biotin conjugate (2 µg /ml; 45 min.) was detected by streptavidin-Cy3 (1.2 µg / ml; 15 min; Jackson laboratories). After washing, FAST-BLUE (Sigma) was used as substrate for the alkaline phosphatase (10-30 min). For all slides the procedure was identically performed with isotype controls.
EMMPRIN was detected on 82% of 140 cytokeratin-positive tumor cells derived from 68 patients with breast, prostate and lung cancer (Tab. 8 and Fig. 20). In only two aspirates all detected cytokeratin-positive cells (n=4) were negative for EMMPRIN.

Table 8. EMMPRIN (EMM) protein expression on disseminated cytokeratin-positive (CK+) tumor cells in bone marrow.

Number of patients	number of patients with CK+ cells	Total number of CK+ cells	CK+ / EMM+ cells	Number of patients with double positive cells
68	11/68 (16%)	140	115/140 (82%)	9/11 (82%)

Besides Emmprin also expression of transferrin receptor (CD71) on tumor cells was evaluated on protein level. Transcriptome analysis of six small biopsies derived from non-small cell lung cancers and five biopsies of control mucosa from patients with chronic obstructive pulmonary showed that signal intensity for CD71 differed greatly between normal and tumor tissue (Table 9).

Table 9. Signal intensities for the transferrin receptor cDNA on array hybridisation

Tumor biopsies						Normal Mucosa biopsies				
Bio 6	Bio9	Bio10	Bio11	Bio1G	Bio11G	Bio2	Bio3G	Bio5G	Bio6G	Bio14
0	2464	11768	4012	0	5496	0	0	0	100	0

Differential expression was tested on cryosection of tumor biopsy Bio10 and a biopsy from normal mucosa (Bio6G). Unspecific binding was blocked with 10% AB serum in PBS for 20 minutes and incubation with CD71-PE (phycoerythrin) conjugated antibody (Caltag) was performed for 45 minutes. For control an anti-CD4-PE antibody was used. No staining of the CD4 antibody was observed on either tissue sample. The CD71-PE antibody selectively stained the epithelial regions of the tumor biopsy whereas the normal mucosa was negative for transferrin receptor expression (Fig. 21).

Example X: Anti-apoptotic effect of IFNγ on smooth muscle cells

The effect of IFNγ on the survival of cultured proliferating SMCs was analyzed by flow cytometry. For this reason primary human coronary smooth muscle cells were obtained from CellSystems (Germany) and were grown in Smooth muscle cell growth medium (CellSystems) containing 5% fetal calf serum (CellSystems) at 37°C in a humidified atmosphere of 5% CO_2. SMCs were used between passages 2 and 4. Treatment with 1000U/ml rh-IFNγ (R&D Systems) was performed for 16h. For induction of cell death, SMCs were incubated at 37°C for 1h in HBSS containing 100 µmol/l H_2O_2 and 100 □mol/l ferrous sulfate. Afterwards the cells were further cultured in freshly prepared culture medium for 8h. Cells were labelled with FITC-labelled Annexin V (Roche Diagnostics) and propidium iodide (PI) according to the manufacturer's instructions. 10^4 events were analyzed with a flow cytometer (Becton Dickinson).

Flow cytometric analysis revealed an anti-apoptotic effect of IFNγ on SMCs (Fig. 22). FACS analysis after double staining with PI and FITC-labeled Annexin V and showed a reduction of spontaneous apoptosis from 10% to 6% after treatment with IFNγ. The effect became more prominent after induction of apoptosis in SMCs with H_2O_2. Treatment with IFNγ reduced the number of apoptotic cells from 54% to 27%. These results clearly show that IFNγ exerts an anti-apoptotic effect on SMCs.

Example XI: Inhibitory effect of IFNγ on neointima formation in a mouse model for restenosis

To examine the vascular proliferative remodeling after carotid ligation, the mouse blood flow cessation model (Kumar, Circulation 96 (1997), 4333-4342) was used. This model is characterized by vascular proliferation of SMCs in response to ligation of the common carotid artery near bifurcation. In order to investigate the effect of an IFN-γ receptor null mutation on the development of neointima in a mouse model of restenosis IFN-γ$R^{-/-}$ knockout mice were used. Adult male 129/svJ mice (N=16) and IFN-γ$R^{-/-}$ mice (n=11) were anaesthetized by intraperitoneal injection of a solution of xylazine (5 mg/kg body weight) and ketamine (80 mg/kg body weight) and the left common carotid artery was

ligated near bifurcation. After 4 week animals were reanaesthetized, sacrificied and fixed for 3 min by perfusion with 4% paraformaldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.3,). After excision of the left carotid arteries, vessels were fixed by immersion in 70% ethanol. Carotid arteries were embedded in paraffin and serial sections (%µm thick) were cut.

Morphometric analysis was performed on v.-Giesson stained cross sections at a distance of 600 µm from the ligation site. Digitized images of the vessels were analyzed using the image analysis software SCION image 4.0.2. Media thickness was obtained as the differences in diameter between the external and internal elastic lamina, and neointima thickness as the difference between internal elastic lamina and lumen diameter. Data from morphometric analyses are reported as mean ± SEM for the two groups of mice and tested by the t-test for unpaired samples. A p value < 0.05 was regarded as significant. All analyses were performed with the use of the SPSS statistical package (version 8.0).

Substantial wall thickening due to media proliferation and neointima formation was observed in 16 wild-type mice at 4 weeks after ligation (Fig. 23). In 11 IFN-$\gamma R^{-/-}$ mice medial plus neointimal thickening was significantly reduced shown as mean ± SEM and analyzed by the t-test for unpaired samples. Corresponding to the reduction in proliferative responses, 11 IFN-$\gamma R^{-/-}$ mice had a significantly larger lumen diameter of the treated carotid segment than wild-type mice (108±15 um versus 91±24 um and p=0.033).

Example XII: Suppression subtractive hybridization (SSH) analysis

SSH is a new and highly effective method for the generation of subtracted cDNA libraries. Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Duguin Nucl. Acid. Res. 18 (1990), 2789-2792 and Hara Nucl. Acid. Res. 19 (1991), 7097-7104 and Hendrick Nature (London) 308 (1984), 149-153). In general, hybridization of cDNA from one population (tester) to an excess of mRNA (cDNA) from another population (driver) and subsequent separation of the unhybridized fraction (target) from the hybridized common sequences are performed. SSH is used to selectively amplify target cDNA fragments (differentially

expressed) and simultaneously suppress nontarget DNA amplification. The method is based on suppression PCR: long inverted terminal repeats when attached to DNA fragments can selectively suppress amplification of undesirable sequences in PCR procedure. The problem of differences in mRNA abundance is overcome by a hybridization step that equalizes sequence abundance during the course of subtraction. One subtractive hybridization round is required leading to a 1000 fold enrichment for differentially expressed cDNAs (for review see Diatchenko Proc. Natl. Acad. Sci. USA 93 (1996), 6025-30 and Diatchenko Methods Enzymol. 303 (1999), 349-80).

Serveral modifications were introduced into the standard SSH protocol for differential gene expression analysis of a very small number of cells (Fig. 24). 1) mRNA amplificates generated according to the method described in this patent application had been reverse-transcribed and amplified using CP2 primers; 2) mRNA amplificates generated according to the method described in this patent application themselves form panlike structures; 3) introduction of a restriction enzyme recognition site (e.g. EcoRI) into the CP2 primer.

a) Materials and Methods

Oligonucleotides

cDNA synthesis primer :

CP2 : 5'-TCA GAA TTC ATG CCC CCC CCC CCC CCC C-3' (SEQ ID NO: 14)

Adapters

Adapter 1 (A1)

Eco 44 I : 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CTC GCC CGG GCA GG-3' (SEQ ID NO: 31)

Eco 12 I : 5'-AAT TCC TGC CCG-3' (SEQ ID NO: 32)

Adapter 2 (A2)

Eco 43 II : 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT GGT GCG GAG GGC G-3' (SEQ ID NO: 33)

Eco 12 II : 5'-AAT TCG CCC TCC-3' (SEQ ID NO: 34)

PCR Primers :

P1-30 : 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG-3' (SEQ ID NO: 35)

P2-30 : 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT-3' (SEQ ID NO: 36)

P1-33: 5'-GTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CTC-3' (SEQ ID NO: 37)

P2-33: 5'-TGT AGC GTG AAG ACG ACA GAA AGG TCG CGT GGT-3' (SEQ ID NO: 38)

PN1-30 : 5'-CGA CTC ACT ATA GGG CTC GAG CGG CTC GCC-3' (SEQ ID NO: 39)

PN2-30 : 5'-GTG AAG ACG ACA GAA AGG TCG CGT GGT GCG-3' (SEQ ID NO: 40)

Driver Preparation

For detection of transcripts differentially expressed in micrometastatic tumor cells compared to normal bone marrow cells, driver was prepared from bone marrow samples derived from healthy donors. From three bone marrow donors, total RNA was isolated using standard protocols. RNA corresponding to 300.000 bone marrow cells was then added to 30 µl Dynal beads and the protocol of mRNA amplification was performed.

Hybridization kinetics were improved by digestion of 5µg driver with 50 units of restriction enzyme Rsa I in a 50µl reaction containing 0,75 x buffer NEB1 (New England Biolabs) for 90 min. The sample was desalted with a Microcon 10 column (Millipore).

Tester preparation

Eco RI digested tester was prepared in 50 µl using 50 U EcoRI. As tester a mixture of four single cells isolated from four different breast cancer patients was selected. After digestion with EcoRI the tester was diluted to a 100 ng/µl concentration in water. Subsequently, one probe was ligated to 5 µl of adapter A1 (SEQ ID NO: 31, SEQ ID NO: 32) and one to adapter A2 (SEQ ID NO: 33, SEQ ID NO: 34) (50 µM) in two independent 10 µl ligation reactions at 15°C overnight, using 5 units of T4 DNA ligase (Roche). The ligation reaction was inactivated by addition of 2 µl 0.1 M EDTA and heating 5 min at 70°C.

Subtractive hybridization

1 µl of driver (500 ng) was added to each of two tubes containing 2 µl of tester cDNA (about 18 ng) ligated to adapter A1 (SEQ ID NO: 31, SEQ ID NO: 32) and ligated to adapter A2 (SEQ ID NO: 33, SEQ ID NO: 34) in hybridization buffer (1 M NaCl, 50 mM Hepes, 1 mM CTAB). The solution was overlaid with mineral oil, denatured 1 min at 98°C and then allowed to anneal for 10-14 hours at 68°C.

After the first hybridization, both samples were mixed together and about 150 ng heat-denatured driver in 1.5 µl hybridization buffer were added. The sample is allowed to hybridize for 10-14 hours. Hybridization was stopped by adding 200 µl of dilution buffer (20 mM Hepes, pH 8.3, 50 mM NaCl, 0.2 mM EDTA) and by heating for 7 min at 68°C.

PCR amplification

Two PCR amplification reactions were carried out for each subtraction in a volume of 25 µl. First PCR was performed in Taq long template buffer 1 (Roche) with 1 µl of diluted, subtracted cDNA, 1 µl PCR primer P1-30 (SEQ ID NO: 35) (8 µM) and 1 µl primer P2-30 (SEQ ID NO: 36) (8 µM) and 0.4 mM dNTPs. Taq polymerase was added in a hot start procedure. The PCR-cycler was set to 75°C for 7 min (filling in the ends), 27 cycles were performed (94°C, 30 sec ; 66°C, 30 sec ; 72°C, 1.5 min) and a final extension at 72°C for 7 min. PCR products were diluted 10 fold in water and 1 µl was used for a secondary PCR performed according to the protocol described above, but using PCR primers PN1-30 (SEQ ID NO: 39) and PN2-30 (SEQ ID NO: 40) and 12 cycles (94°C, 30 sec.; 68°C, 30 sec ; 72°C, 1.5 min). PCR products were analyzed by gel electrophoresis on a 1.5 % agarose gel.

Cloning and analysis of subtracted cDNA

Products from secondary PCR were ligated into the pGEM-Teasy, a T/A cloning system (Promega). After selection of clones with X-Gal/IPTG/ampicilline, inserts were screened by PCR using PN1-30 (SEQ ID NO: 39) and PN2-30 (SEQ ID NO: 40) primers. Differential expression was verified by southern blot analysis of the amplified inserts using labeled tester and driver as probes. Labeling of the driver and tester samples was identical to the labeling for array analysis.

Differentially hybridizing clones were subjected to plasmid preparation using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced. Nucleic acid homology search was performed using the BLAST program (NCBI).

Results

PCR amplification was performed with primer sets of different length (30 nucleotides: P1-30 (SEQ ID NO: 35), P2-30 (SEQ ID NO: 36) and 33 nucleotides: P1-33 (SEQ ID NO: 37) and P2-33 (SEQ ID NO: 38)) both leading to comparable results. Most preferable were primers consisting of 30 nucleotides (P1-30 (SEQ ID NO: 35) and P2-30 (SEQ ID NO: 36)). Smaller primers with 22 nucleotides (Clonetech) as described by Diatchenko (Proc. Natl. Acad. Sci. USA 93 (1996) did not work in PCR reaction. After subtraction, colonies were screened by PCR and the products were subjected to gel electrophoresis and blotting. Labeled tester and driver were hybridized onto the blot as shown for one example in Fig. 25. Colony #4 was identified as a transcription factor described as epithelium-specific gene (Oettgen Genomics 445, (1997) 456-457 and Oettgen Mol. Cell. Biol. 17 (1997), 4419-4433) and Oettgen Genomics 55 (1999), 358-62. This result was confirmed by PCR using the samples from which driver and tester had been prepared (Figure 26).

Claims

1. A method for the amplification of mRNA of a sample, comprising the steps of

   i. generating cDNA from polyadenylated RNA employing at least one primer hybridizing to said polyadenylated RNA and comprising a 5' poly(C) or a 5' poly(G) flank;

   ii.(aa) if present, removing non-hybridized, surplus primer(s) and/or surplus dNTPs;

   (ab) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed; or

   (b) 3' tailing of said generated cDNA with a poly(G) tail when in step i. (a) primer(s) comprising a 5' poly(C) flank was/were employed or a poly(C) tail when in step i. (a) primer(s) comprising a 5' poly(G) flank was/were employed using an RNA-ligase, irrespective of the presence or absence of surplus primer(s) and/or surplus dNTPs; and

   iii. amplifying the tailed cDNA with a primer hybridizing to the tail(s) generated in step ii(ab) or ii(b).

2. The method of claim 1, wherein said at least one primer in step "i" is a random primer, a oligo(dT) primer or a combination thereof.

3. The method of claim 2, wherein said random primer comprises a random hexamer or a random octamer oligonucleotide.

4. The method of claims 2 or 3, wherein said random primer has a sequence as shown in SEQ ID NOs: 1-9.

5. The method of claim 2, wherein said oligo(dT) primer has the sequence as shown

in SEQ ID NO: 10.

6. The method of any one of claims 1 to 5, wherein in step "i" the concentration of said at least one primer is in the range of 0.01 μM to 500 μM preferably in the range of 0.1 μM to 200 μM, more preferably in the range of 1 μM to 100 μM, even more preferably in the range of 10 μM to 60 μM.

7. The method of any one of claims 1 to 6, wherein said primer in step "iii" comprises a stretch of at least 10, preferably at least 12, most preferably at least 15 nucleotides capable of hybridizing with the tail(s) generated in step "ii(ab)" or "ii(b)".

8. The method of claim 7, wherein said primer has the sequence as depicted in SEQ ID NO: 11, 12, 13, 14 or 15.

9. The method of any one of claims 1 to 8, wherein said polyadenylated RNA is bound to a solid support.

10. The method of claim 9, wherein said solid support is a bead, a membrane, a filter, a well, a chip or a tube.

11. The method of claim 10, wherein said bead is a magnetic bead, a latex bead or a colloid metal bead.

12. The method of claim 10 or 11, wherein said bead comprises an oligo(dT) stretch.

13. The method of any one of claims 1 to 12, wherein said mRNA is derived from a tissue, a low number of cells or a single cell.

14. The method of claim 13, wherein said low number of cells is in a range of 10^6 to 2 cells.

15. The method of claim 13 or 14, wherein said tissue, cells or single cell is of plant or animal origin.

16. The method of claim 15, wherein said animal is human.

17. The method of any one of claims 13 to 16, wherein said tissue, low number of cells or single cell is a chemically fixed tissue, chemically fixed low number of cells or chemically fixed cell.

18. The method of any one of claims 13 to 17, wherein said tissue, low number of cells or single cell is derived from a body fluid or from solid tissue.

19. The method of any one of claims 1 to 18 further comprising a step "iv" wherein the generated amplified cDNA is further modified.

20. The method of claim 19, wherein said modification comprises the introduction of means for detection.

21. The method of claim 20, wherein said means of detection comprises the introduction of nucleotide analogues coupled to (a) chromophore(s), (a) fluorescent dye(s), (a) radio-nucleotide(s), biotin or DIG.

22. The method of any one of claims 1 to 21, wherein the obtained amplified cDNA is bound to a solid support.

23. The method of any one of claims 1 to 22, wherein all or individual steps are carried out in a non-cacodylate buffer.

24. The method of claim 23, wherein said non-cacodylate buffer is a phosphate buffer.

25. The method of claim 24, wherein said phosphate-buffer is a KH_2PO_4 buffer.

26. The method of any one of claims 1 to 25 wherein said sample is derived from a cell and/or a tissue, the genetic identity of which had been defined by comparative genomic hybridzation.

27. A method for the preparation of an in vitro surrogate for (a) pathologically modified cell(s) or tissue(s) comprising the steps of:
    (a) amplifying mRNA of said pathologically modified cell(s) or tissue(s) according to the steps of the method of any one of claims 1 to 26;
    (b) assessing the quantity and, optionally, biophysical characteristics of the amplified cDNA and/or transcripts thereof, thereby determining the gene expression pattern of said pathologically modified cell(s) or tissue(s);
    (c) selecting an in vitro cell, the gene expression pattern of which resembles the gene expression pattern of said pathologically modified cell(s) or tissue(s); and
    (d) adapting the gene expression pattern of said in vitro cell to the gene expression pattern of the pathologically modified cell or tissue.

28. The method of claim 27 further comprising the steps of
    b(1). determining the gene expression pattern of (a) control cell(s) or (a) control tissue(s); and
    b(2). determing the gene(s) which is/are differentially expressed in said for pathologically modified cell or tissue and said control cell or tissue

29. The method of claim 28, wherein said gene expression pattern of a control cell or a control tissue is determined employing the method of any one of claims 1 to 26.

30. The method of any one of claims 27 to 29, wherein said pathologically modified cell or tissue is a restenotic cell or restenotic tissue.

31. The method of any one of claims 28 to 30, wherein said control cell(s) or tissue(s) is/are selected from the group consisting of smooth muscle cells, media/intima of healthy coronary arteries.

32. The method of any one of claims 27 to 31, wherein said in vitro cell is or is derived form a primary cell culture, a secondary cell culture, a tissue culture or a cell line.

33. The method of claim 32, wherien said in vitro cell is selected from the group consisting of cultured coronary artery smooth muscle cells, HepG2 cells, Jurkat cells, THP-I cells, Monomac-6-cells, U937 cells, ATCC 45505 cells, cultured cardiomyocytes, ECV 304 cells and NIH3T3 cells.

34. The method of any one of claims 27 to 33 wherein said adaptation in step c. comprises the exposure of said in vitro cell to physical and/or chemical changes.

35. The method of claim 34, wherein said physical changes comprise temperature shifts, light changes, pH-changes, changes in ionic strength or changes in the gas phase.

36. The method of claim 34, wherein said chemical changes comprise medium exchanges, medium substitutions, medium depletions and/or medium additions.

37. The method of claim 34, wherein said chemical changes comprise the exposure to compounds including growth factors, hormones, vitamines, antibodies or fragments and/or derivatives thereof, cytokines, transcription factors, kinases, antibiotics, natural and/or non-natural receptor ligands and components of signal transduction pathways.

38. The method of claim 37, wherein said cytokine is IFN-γ (or a functional derivative thereof), said natural and/or non-natural receptor ligand is a ligand for IFN-γ receptor (a and/or b chain), said transcription factor is IRF-1 or ISGF3-γ-(p48),

said kinase is tyrosine kinase Pyk2, said components of signal transduction pathways is Dap-1, BAG-1, Pim-1 or IFN-γ-inducible protein 9-27, said growth factor is platelet growth factor AA, angiotension or fibroblast growth factor or said antibiotic is rapamycin.

39. Use of an in vitro surrogate obtainable by the method of any one of claims 27 to 38 in drug screening methods.

40. A method for identifying differentially expressed genes in a test sample, comprising the steps of
    (a) providing a test sample and a control sample each comprising polyadenylated RNA;
    (b) employing the steps of the method of any one of claims 1 to 26 on said test and control sample; and
    (c) comparing the obtained amplified cDNA of said test sample with the obtained amplified cDNA of said control sample.

41. A method for identifying a drug candidate for prevention or therapy of a pathological condition or a pathological disorder comprising the steps of
    (a) contacting a sample comprising polyadenylated RNA with said drug candidate;
    (b) employing the steps of the method of any one of claims 1 to 26 on said sample; and
    (c) detecting the presence, the absence, the increase or the decrease of particular expressed genes in said sample, wherein the correlation of said presence, absence, increase or decrease with the presence of said drug candidate qualifies said drug candidate as a drug.

42. A method for in vitro detection of a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of
    (a) providing a sample comprising polyadenylated RNA from said subject;
    (b) employing the steps of the method of any one of claims 1 to 26 on said

sample; and

(c) detecting a pathological condition or a susceptibility to a pathological condition based on the presence, the absence, the increase, the decrease or the amount of (a) expressed gene(s) in said sample.

43. Use of the amplified cDNA as obtained by the method of any one of claims 1 to 26 for in vitro and/or in vivo expression.

44. Use of the amplified cDNA as obtained by the method of any one of claims 1 to 26 for the in vitro and/or in vivo preparation of mRNA transcripts.

45. Use of the amplified cDNA as obtained by the method of any one of claims 1 to 26 or the use of mRNA transcripts as defined in claim 42 in hybridization assays, or in interaction assays.

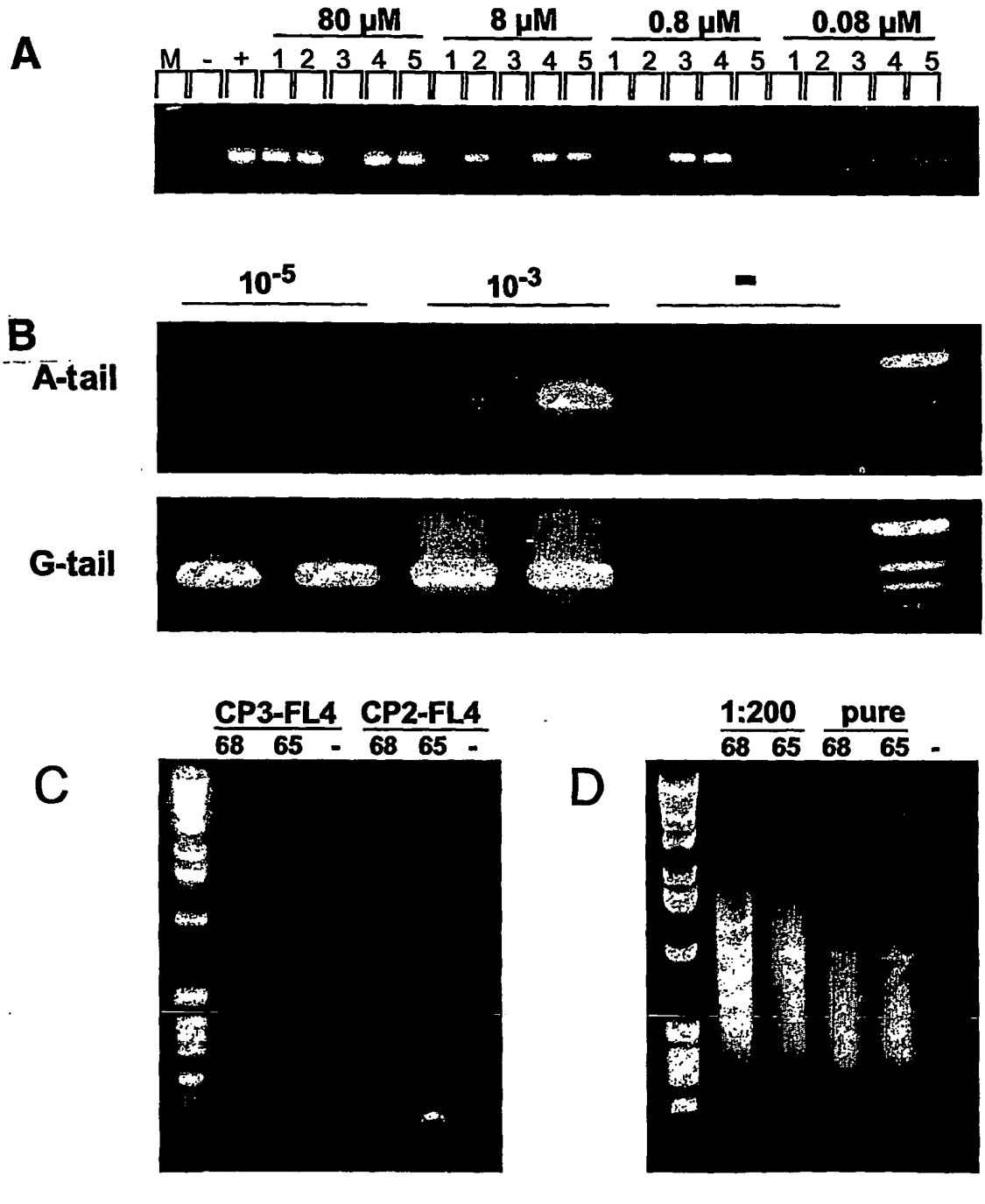
46. The use of claim 44, wherein said hybridization assay comprises the hybridization to oligonucleotide arrays, cDNA arrays, and/or PNA arrays.

47. Use of claim 44, wherein said interaction assay comprises the interaction with carbohydrate(s), lectin(s), ribozyme(s), protein(s), peptide(s), antibody(ies) or (a) fragment(s) thereof, and/or aptamer(s).

48. The use of the amplified cDNA obtained by the method of any one of claims 1 to 26 for sequence specific PCR, cDNA cloning, substractive hybridization cloning, and/or expression cloning.

49. Kit comprising at least one primer as defined in any one of claims 1 to 5 and 7 to 8.

Fig.1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80 µM
8 µM
0.8 µM
0.08 µM
M - + 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
B
10-5
10-3
A-tail
G-tail
C
CP3-FL4 CP2-FL4
68 65 - 68 65 -
D
1:200 pure
68 65 68 65 -

Fig. 2

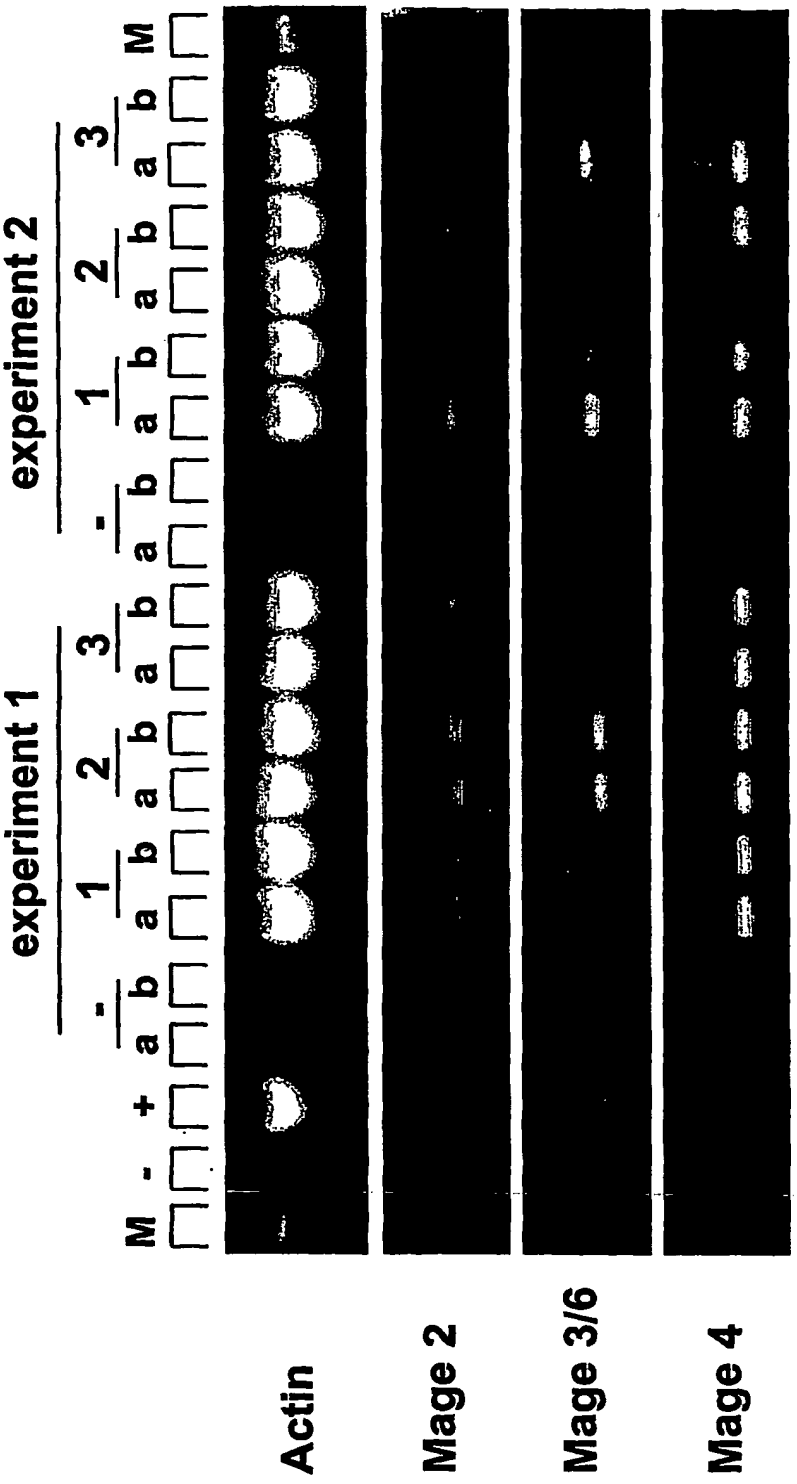

experiment 1
experiment 2
M - + - a b 1 a b 2 a b 3 a b - a b 1 a b 2 a b 3 a b M
Actin
Mage 2
Mage 3/6
Mage 4

Fig. 3

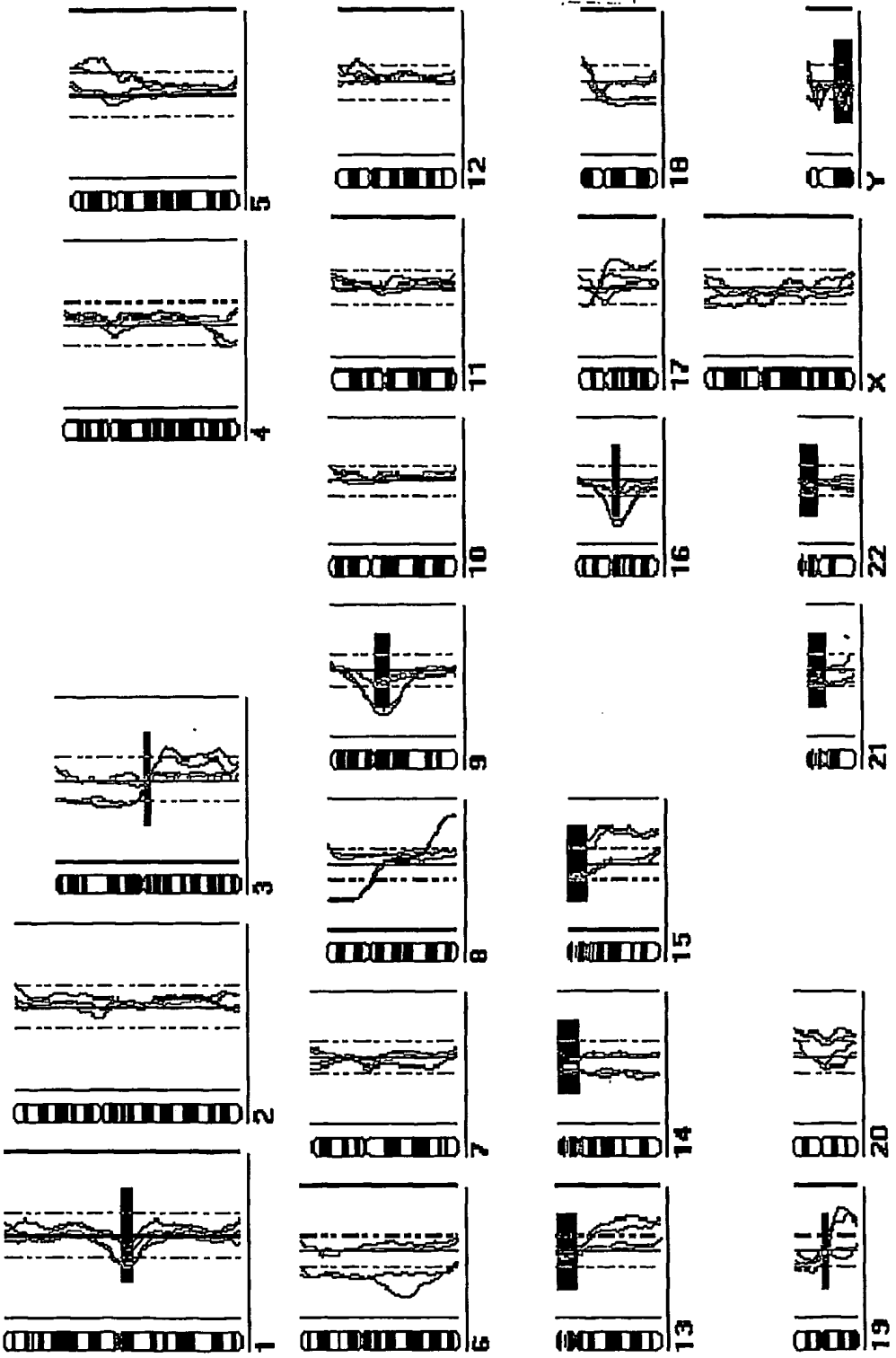


Fig. 4

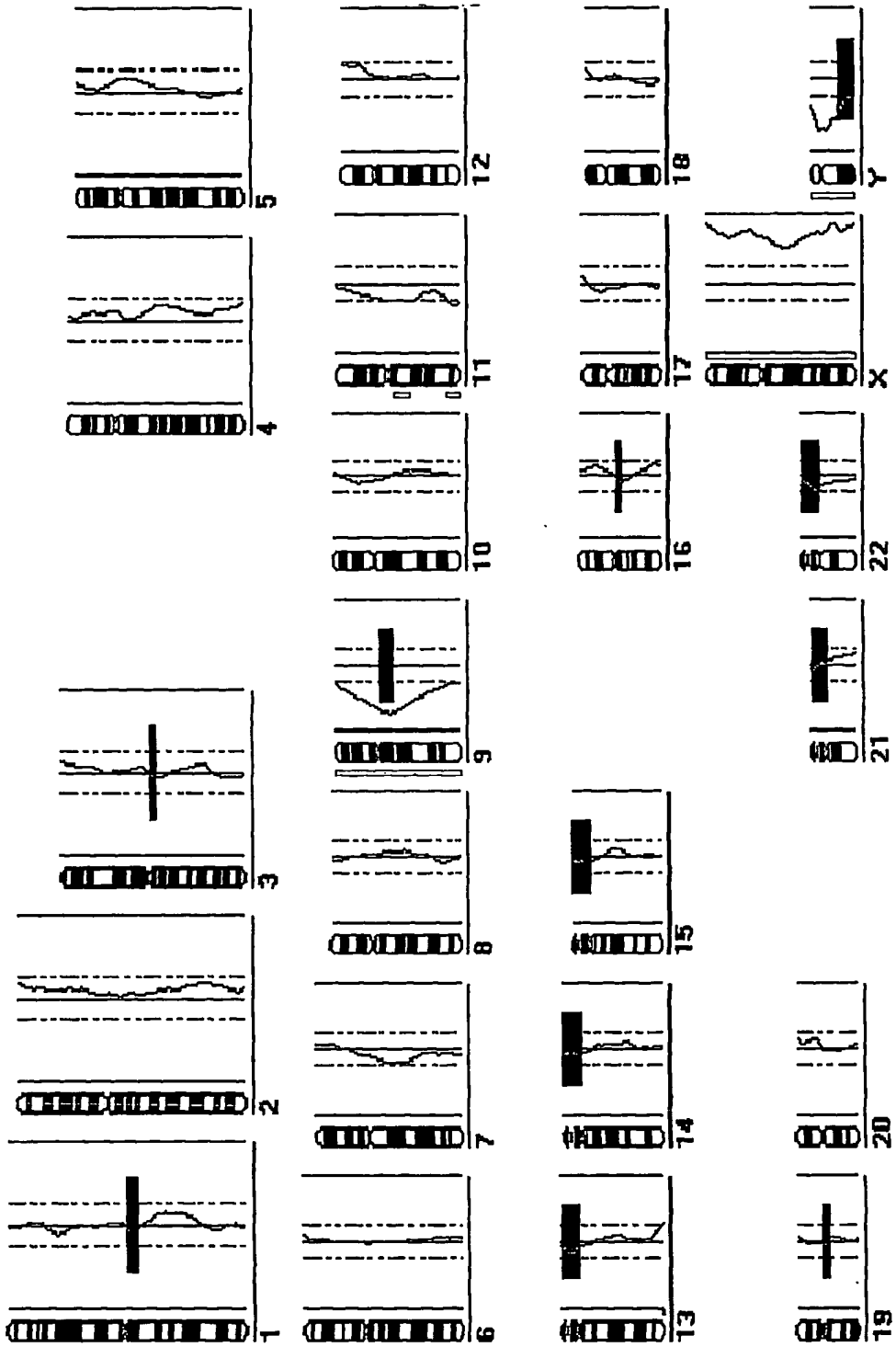


Fig. 5

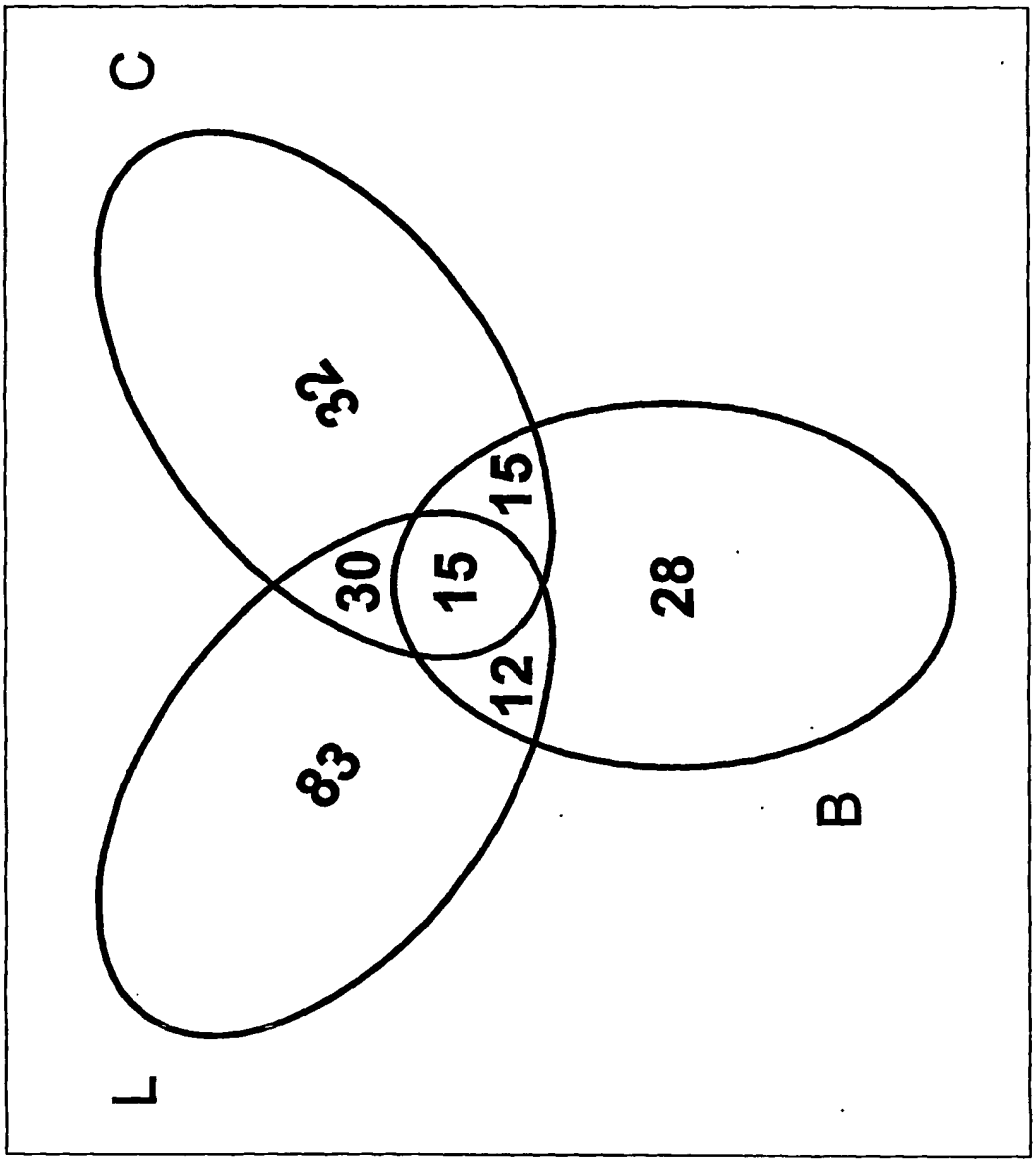


Fig. 6

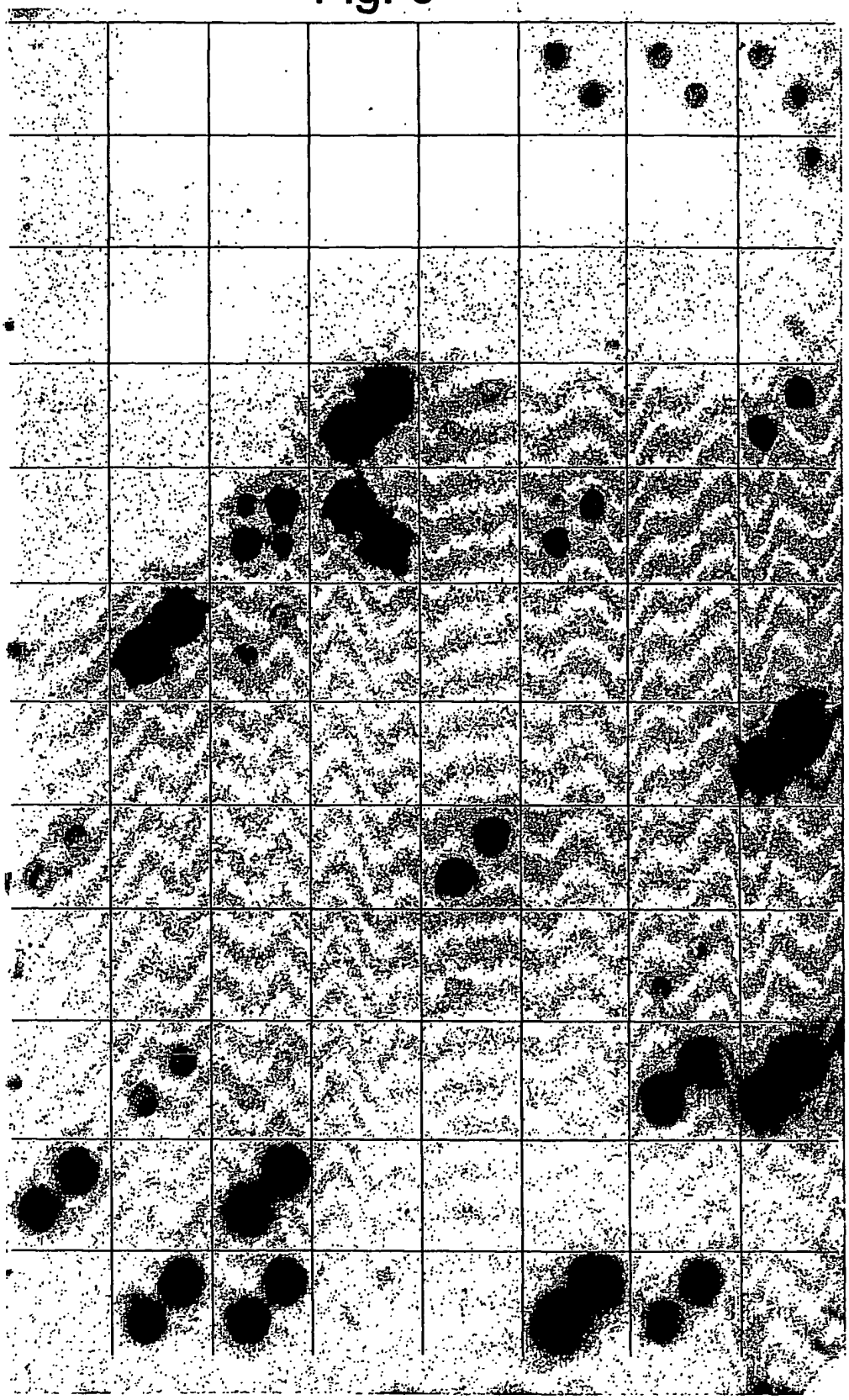


Fig. 6 cont.

GFP	BSG	MMP1	MMP3	ADAM8	ITGA4	ITGB1	CDKN2B PTK2	CCND1 MYC (I)	MAGE A1 THBS1	FCGR3A TGFA	pBS ERBB2
KRT7	MT1MMP	PLAU	MMP11	ADAM9	ITGA5	ITGB2	CDKN2A SLA	GAS1 MYC (II)	MAGEA3 ABCC1	CD33 TGFB1	TCRA TGFBR1
KRT8	MT2MMP	PLAUR	MMP2	ADAM10	ITGA6	ITGB3	CDKN1A P68	MKI67 RB1	MAGEA4 ABCB1	CD34 VEGF	IGKC TGFBR2
KRT10	MT3MMP	PAI1	MMP9	ADAM11	ITGAV	ITGB4	CDKN1B EPHA2	ACTB TK1	MAGEA6 PTPRJ	CD37 IGF1	IGLC1 IGFR1
KRT13	MT4MMP	PAI2	MMP7	ADAM15		ITGB5	ING1 EFNA1	EEF1A1 RAD51	MAGEA12 PTPRM	CD38 RAMP1	VIM IGFR2
KRT18	TIMP1	CTSB	CSTA	ADAM20	GFP	ITGB7	TP53 (I) CDH1	TNFAIP3 NCK1	MAGEA1s CKM	TNFRSF5 RAMP2	M4S1 MCAM
KRT19	TIMP2	CTSD	CSTB	ADAM21			TP53 (II) CDH3	BCL2 pBS	MAGEA2s MAGEA4s	PTPRC BSG (I)	DSP PHLDA1
KRT20	TIMP4	CTSL	CST3	ADAM17	ACTB		CDKN1C CDH2	GHPDH TERT	MAGEA3s MAGEA12s	CD83 GFP	CEA EEF1A1

Fig. 7

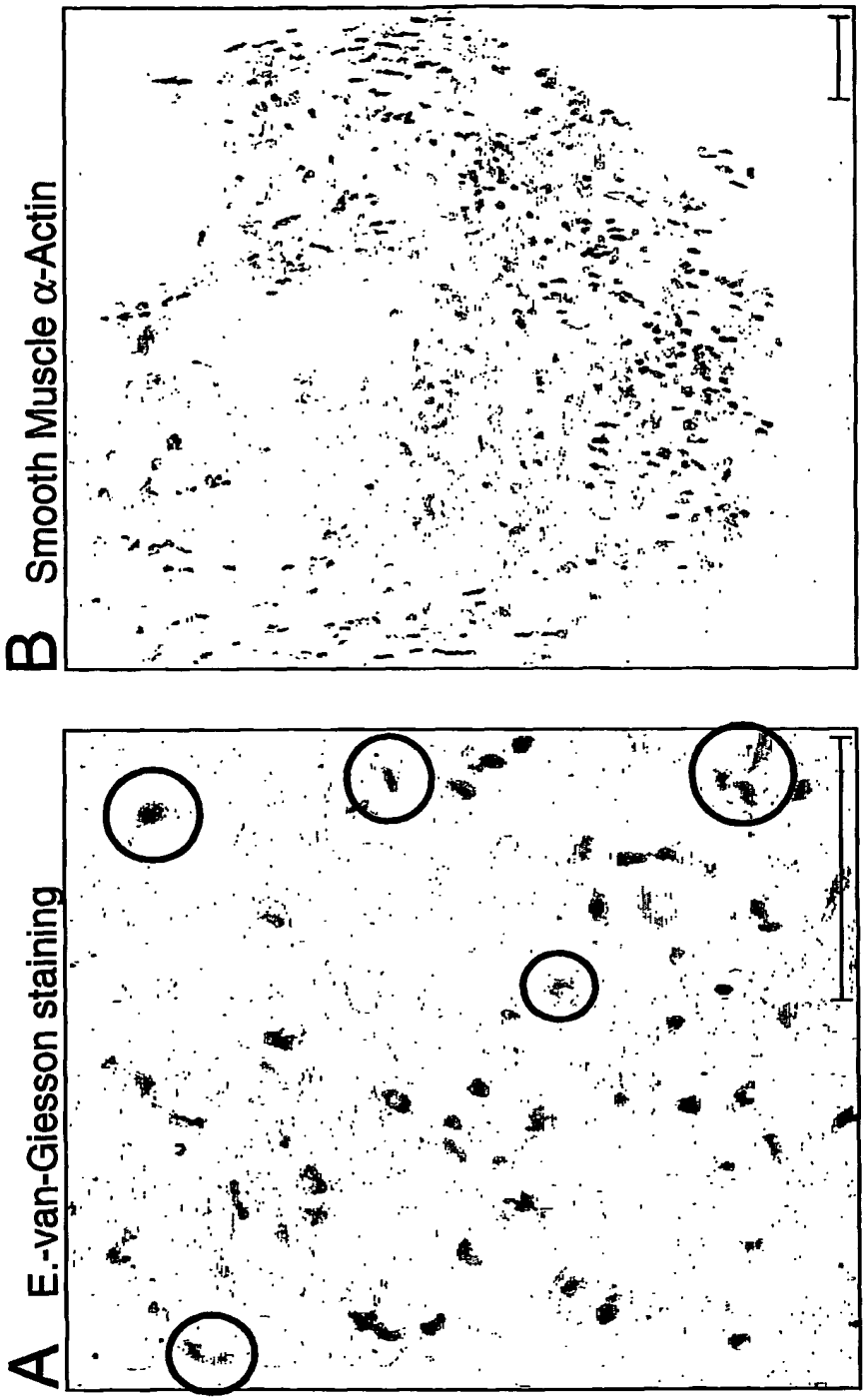


Fig. 8

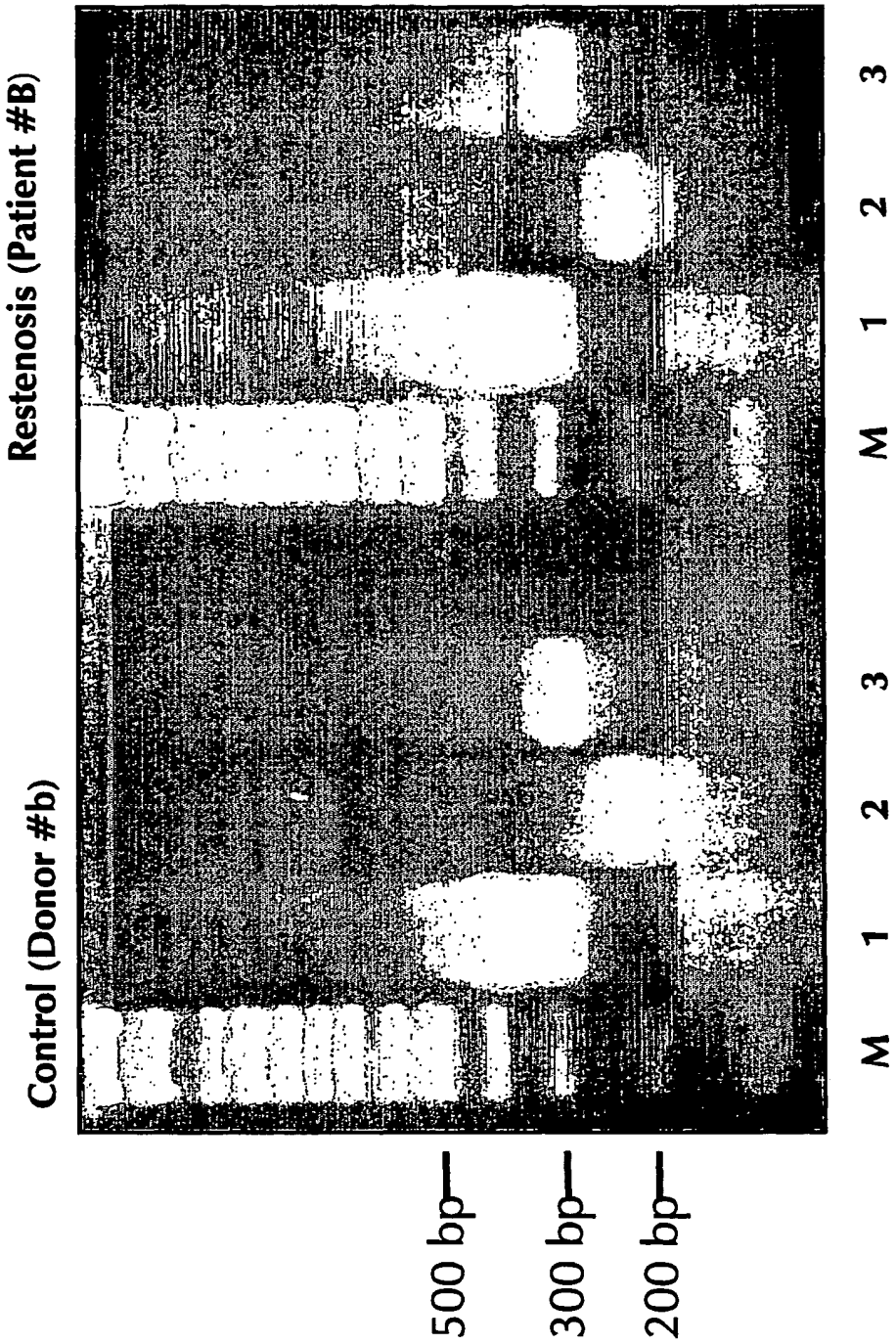
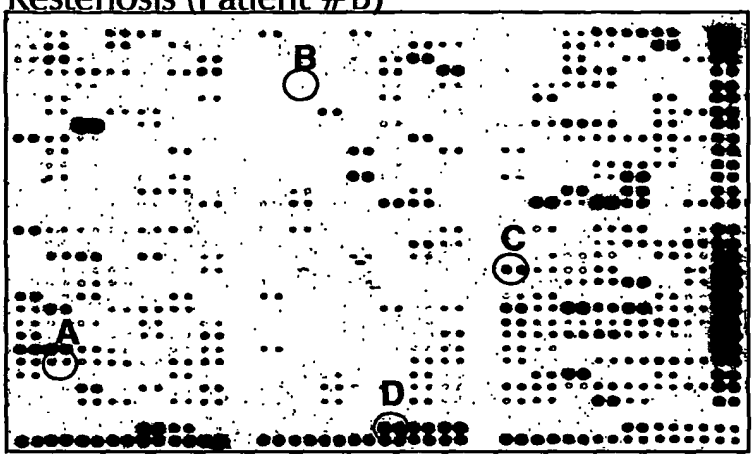

Restenosis (Patient #B)
Control (Donor #b)
M 1 2 3
M 1 2 3
500 bp
300 bp
200 bp

Fig. 9

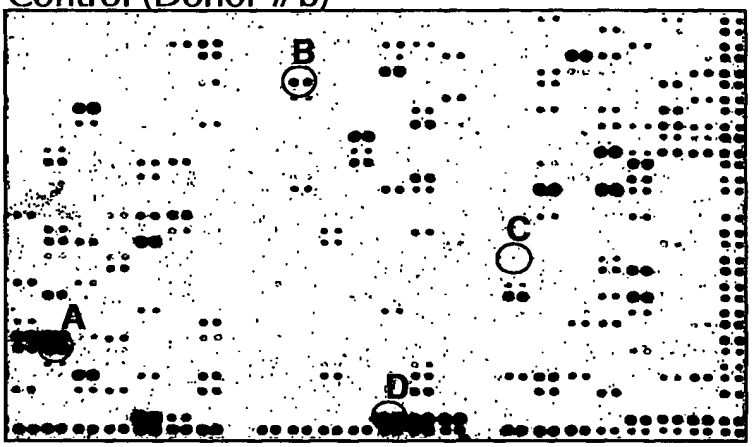
A

Restenosis (Patient #B)



B

Control (Donor #b)



C

Control

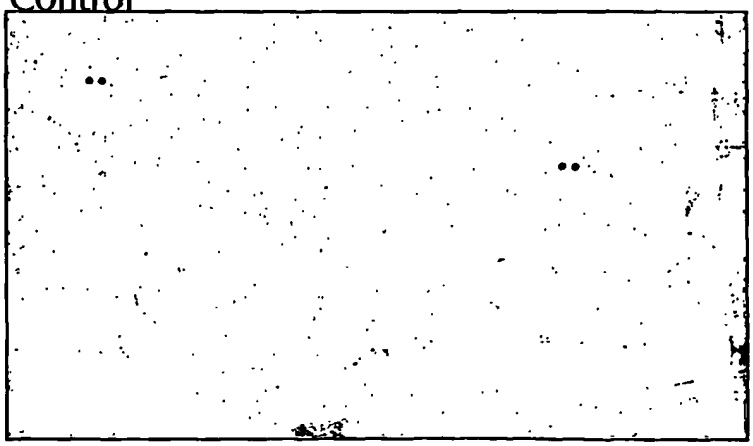
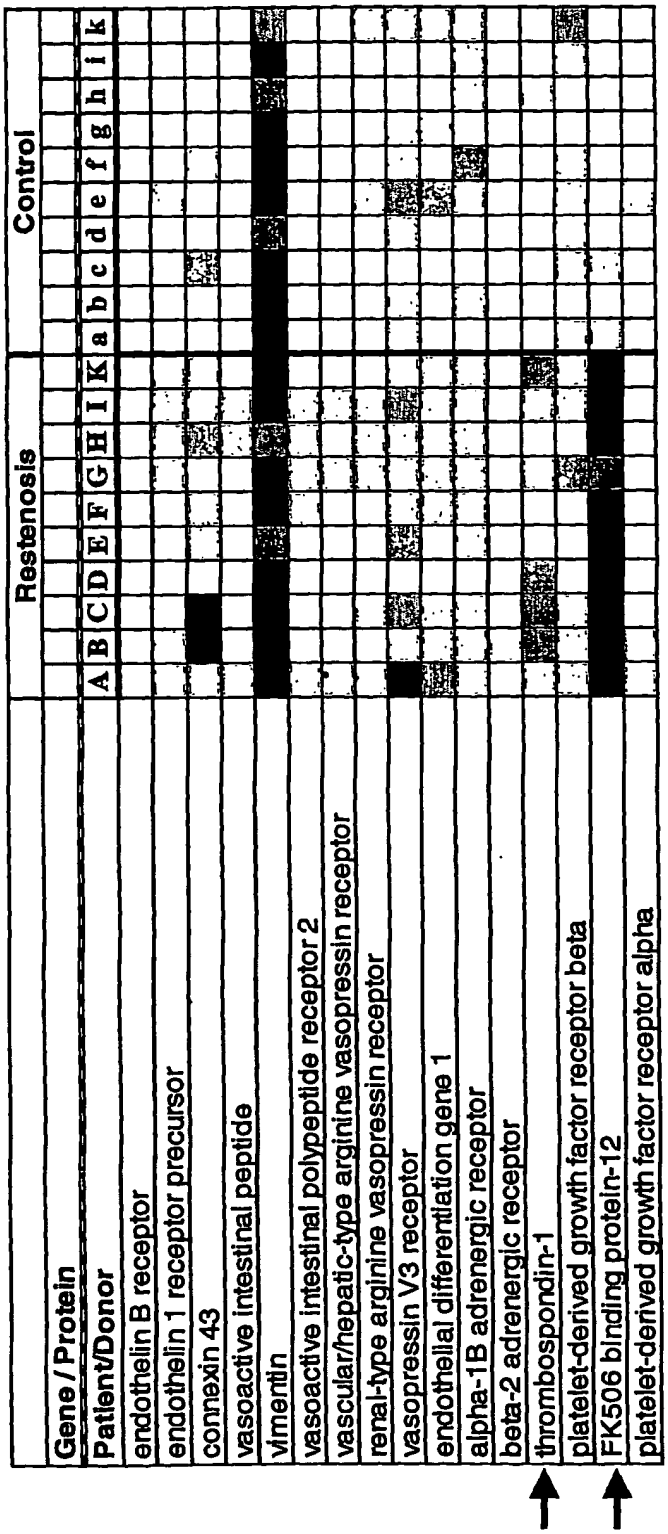


Fig. 10


Restenosis
Control
A B C D E F G H I K a b c d e f g h i k
Gene / Protein
Patient/Donor
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endothelin 1 receptor precursor
connexin 43
vasoactive intestinal peptide
vimentin
vasoactive intestinal polypeptide receptor 2
vascular/hepatic-type arginine vasopressin receptor
renal-type arginine vasopressin receptor
vasopressin V3 receptor
endothelial differentiation gene 1
alpha-1B adrenergic receptor
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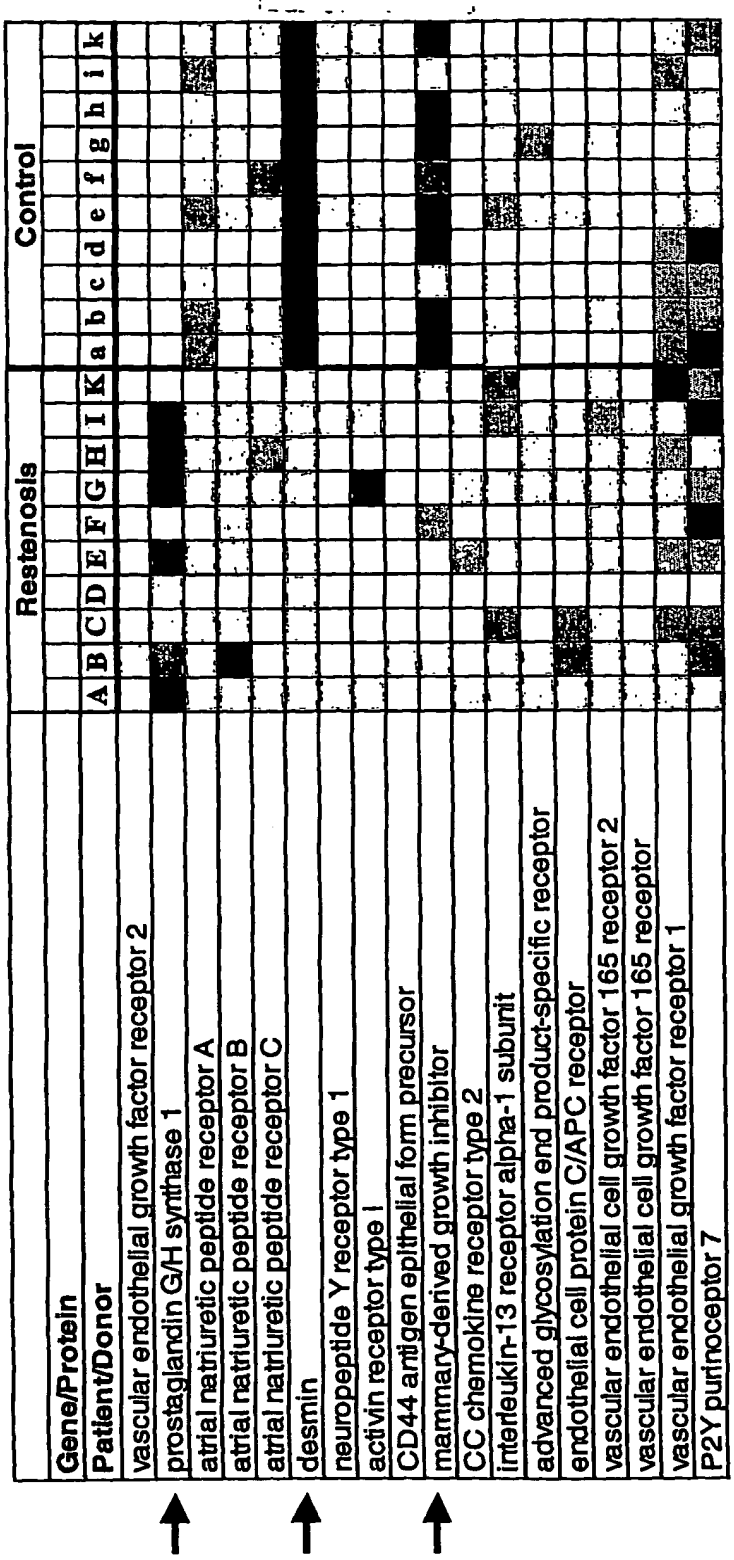
FK506 binding protein-12
platelet-derived growth factor receptor alpha

Fig. 10 cont.


Restenosis
Control
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Gene/Protein
Patient/Donor
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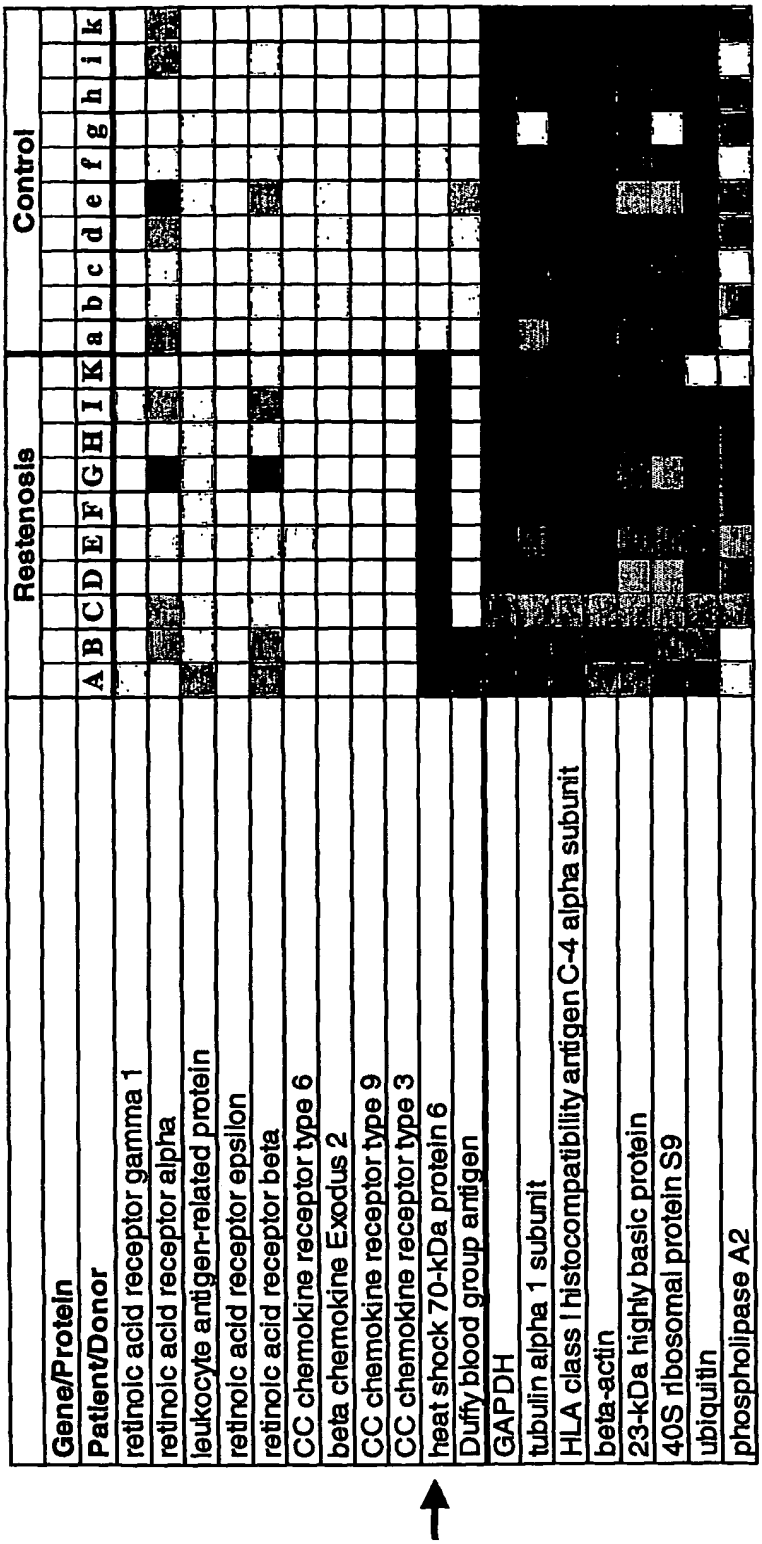
prostaglandin G/H synthase 1
atrial natriuretic peptide receptor A
atrial natriuretic peptide receptor B
atrial natriuretic peptide receptor C
desmin
neuropeptide Y receptor type 1
activin receptor type I
CD44 antigen epithelial form precursor
mammary-derived growth inhibitor
CC chemokine receptor type 2
interleukin-13 receptor alpha-1 suburit
advanced glycosylation end product-specific receptor
endothelial cell protein C/APC receptor
vascular endothelial cell growth factor 165 receptor 2
vascular endothelial cell growth factor 165 receptor
vascular endothelial growth factor receptor 1
P2Y purinoceptor 7

Fig. 10 cont.


Gene/Protein
Patient/Donor
Restenosis
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Control
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retinoic acid receptor alpha
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retinoic acid receptor epsilon
retinoic acid receptor beta
CC chemokine receptor type 6
beta chemokine Exodus 2
CC chemokine receptor type 9
CC chemokine receptor type 3
heat shock 70-kDa protein 6
Duffy blood group antigen
GAPDH
tubulin alpha 1 subunit
HLA class I histocompatibility antigen C-4 alpha subunit
beta-actin
23-kDa highly basic protein
40S ribosomal protein S9
ubiquitin
phospholipase A2

Fig. 11

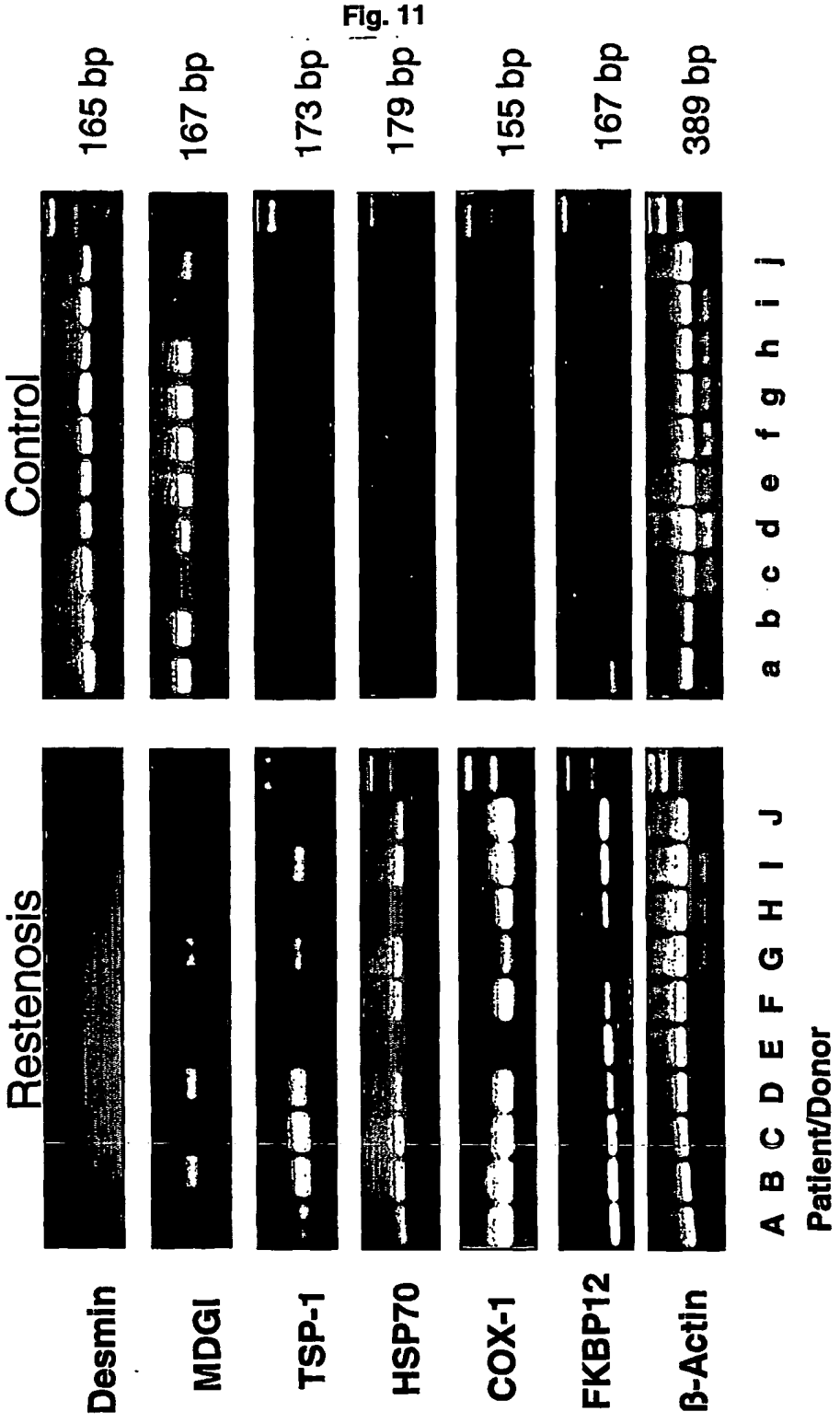

Restenosis
Control
Desmin
MDGI
TSP-1
HSP70
COX-1
FKBP12
β-Actin
165 bp
167 bp
173 bp
179 bp
155 bp
167 bp
389 bp
A B C D E F G H I J
a b c d e f g h i j
Patient/Donor

Fig. 12

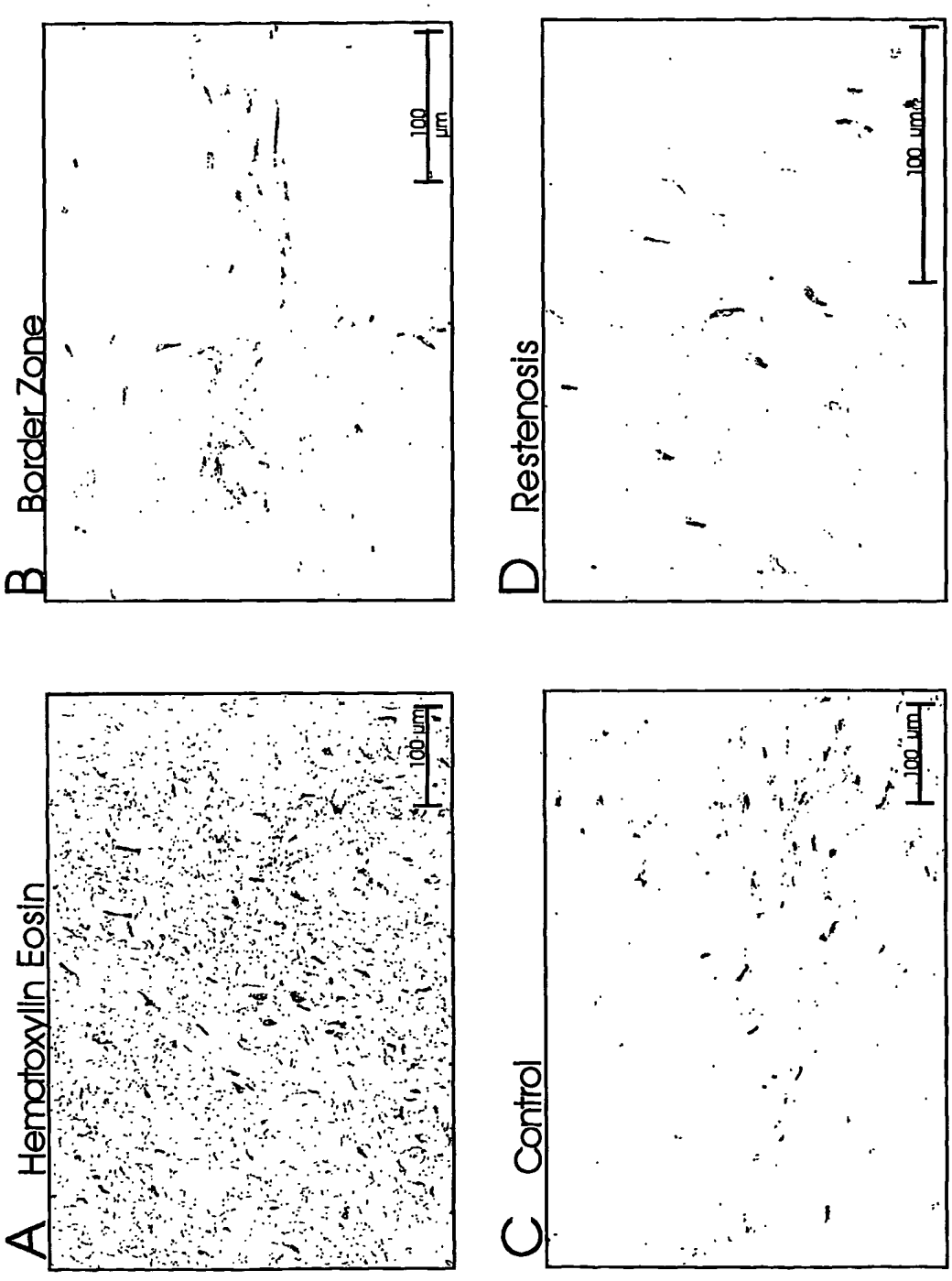
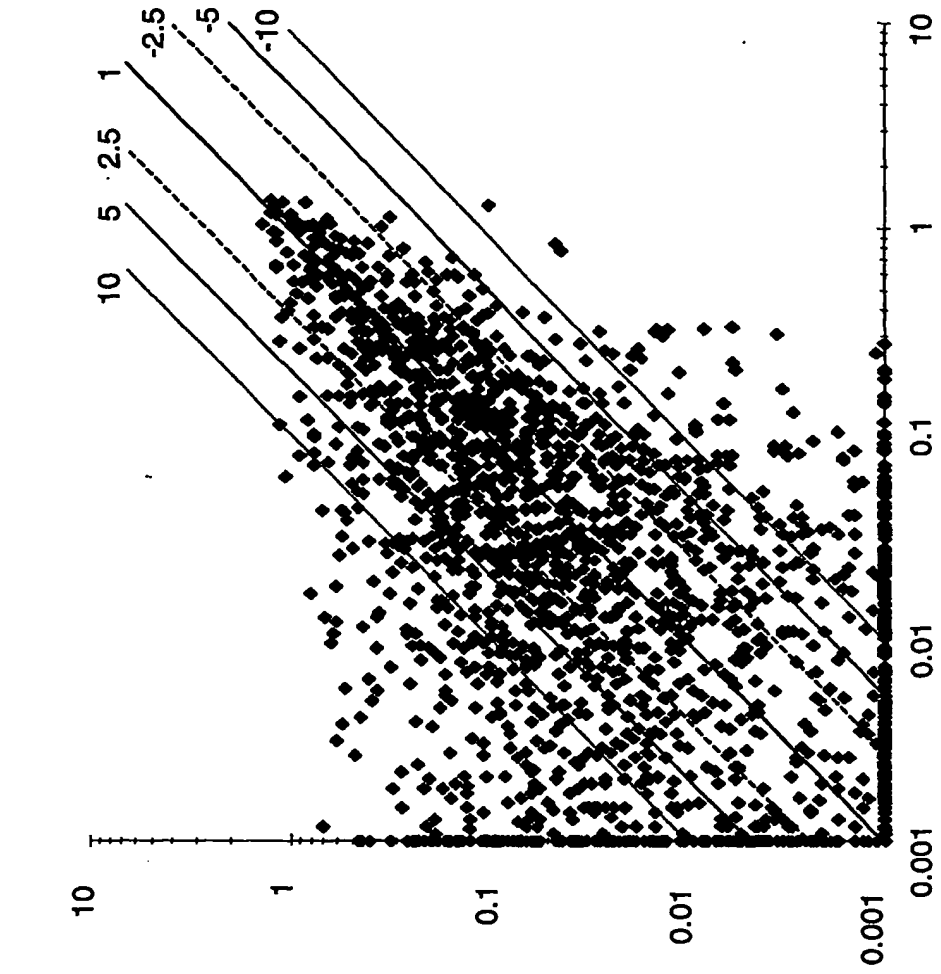

A Hematoxylin Eosin
100 µm
B Border Zone
100 µm
C Control
100 µm
D Restenosis
100 µm

Fig. 13



A

Fig. 13 cont.

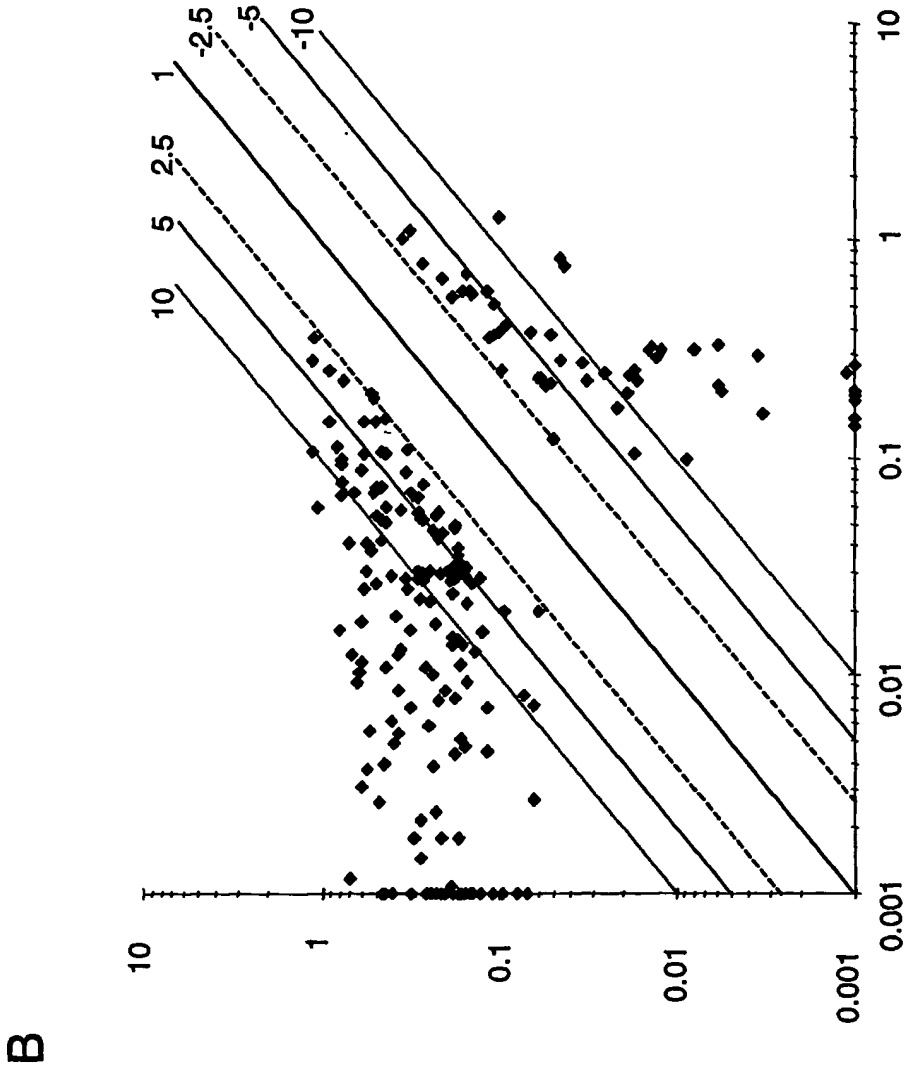
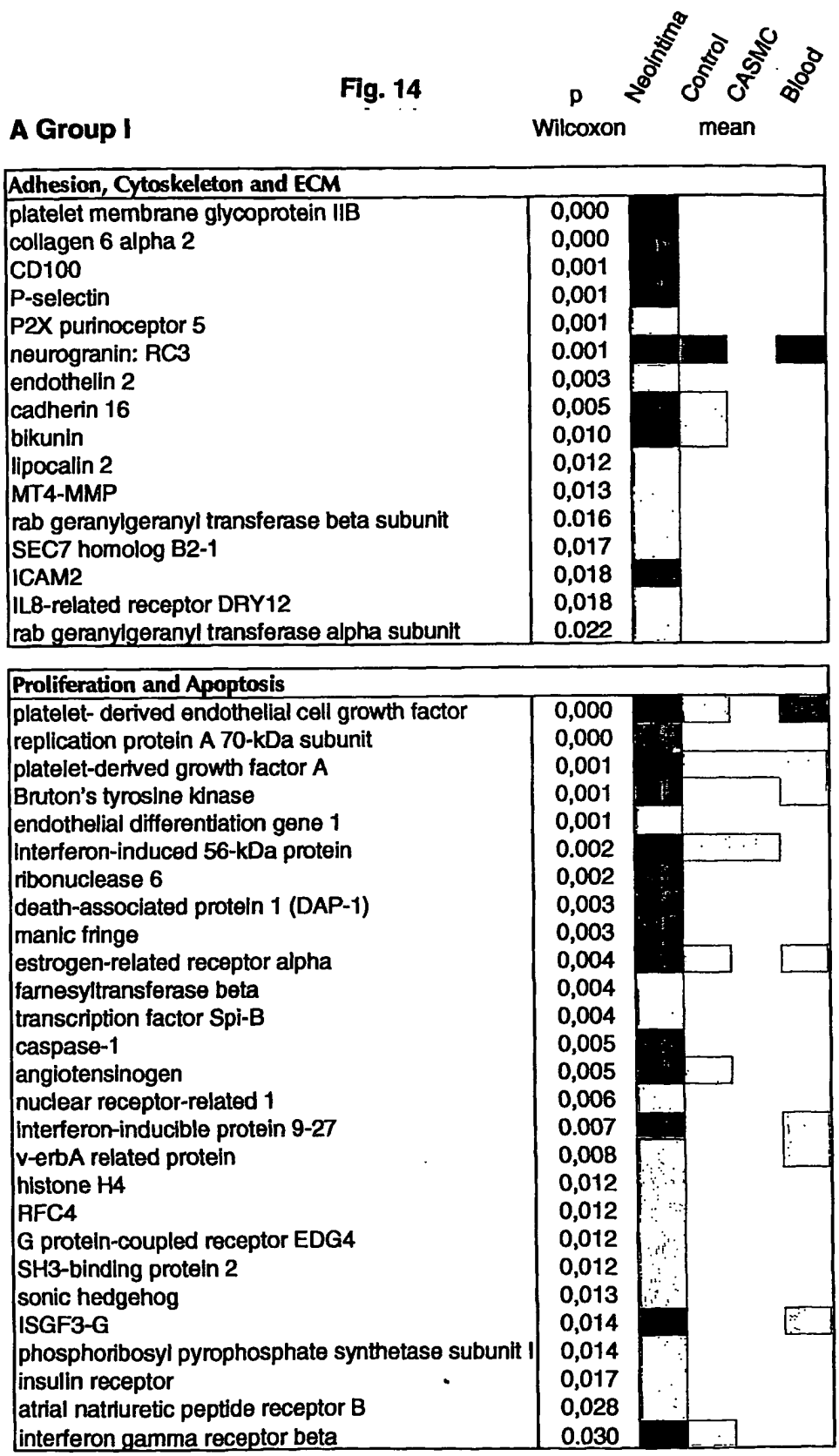


Fig. 14



A Group I

	p Wilcoxon	mean
Adhesion, Cytoskeleton and ECM		
platelet membrane glycoprotein IIB	0,000	
collagen 6 alpha 2	0,000	
CD100	0,001	
P-selectin	0,001	
P2X purinoceptor 5	0,001	
neurogranin: RC3	0.001	
endothelin 2	0,003	
cadherin 16	0,005	
bikunin	0,010	
lipocalin 2	0,012	
MT4-MMP	0,013	
rab geranylgeranyl transferase beta subunit	0.016	
SEC7 homolog B2-1	0,017	
ICAM2	0,018	
IL8-related receptor DRY12	0,018	
rab geranylgeranyl transferase alpha subunit	0.022	
Proliferation and Apoptosis		
platelet- derived endothelial cell growth factor	0,000	
replication protein A 70-kDa subunit	0,000	
platelet-derived growth factor A	0,001	
Bruton's tyrosine kinase	0,001	
endothelial differentiation gene 1	0,001	
interferon-induced 56-kDa protein	0.002	
ribonuclease 6	0,002	
death-associated protein 1 (DAP-1)	0,003	
manic fringe	0,003	
estrogen-related receptor alpha	0,004	
farnesyltransferase beta	0,004	
transcription factor Spi-B	0,004	
caspase-1	0,005	
angiotensinogen	0,005	
nuclear receptor-related 1	0,006	
interferon-inducible protein 9-27	0.007	
v-erbA related protein	0,008	
histone H4	0,012	
RFC4	0,012	
G protein-coupled receptor EDG4	0,012	
SH3-binding protein 2	0,012	
sonic hedgehog	0,013	
ISGF3-G	0,014	
phosphoribosyl pyrophosphate synthetase subunit I	0,014	
insulin receptor	0,017	
atrial natriuretic peptide receptor B	0,028	
interferon gamma receptor beta	0.030	

Fig. 14 cont.

A Group I

	p Wilcoxon	Neointima mean	Control mean	CASMC mean	Blood mean
Inflammation					
vitamin K-dependent protein S	0,000				
alpha-2-antiplasmin	0,001				
coagulation factor XII	0,001				
prothrombin	0,001				
MHC class II HLA-DR-beta	0,002				
CD40	0,011				
interleukin-6 receptor alpha	0,027				
Others					
specific 116-kDa vacuolar proton pump	0,000				
alpha-galactosidase A	0,000				
peroxisomal bifunctonal enzyme	0,000				
glycerol kinase	0,000				
carboxypeptidase N -	0,000				
phenol sulfating sulfotransferase 1	0.001				
apolipoprotein E	0,017				
low-density lipoprotein receptor LR11	0,021				
lysosomal pro-X carboxypeptidase	0,021				
glutathion-S-transferase (GST) homolog	0.026				

Fig. 14 cont.

B Group II

	p Wilcoxon	Neointima mean	Control mean	CASMC mean	Blood mean
Adhesion, Cytoskeleton and ECM					
platelet membrane glycoprotein IIIA	0,000				
migration inhibitory factor-related protein 14	0,000				
amiloride-sensitive epithelial sodium channel ß	0,000				
rho GDP dissociation inihibitor 2	0,000				
paxillin	0,001				
CD13	0,001				
macrosialin	0,001				
p21-rac2	0,003				
CDC42	0.004				
thrombospondin 1	0,007				
versican core protein	0,009				
caveolin 3	0,014				
ICAM1	0,014				
ras-related protein RAB5A	0,014				
calcium & integrin-binding protein	0,018				
cytokeratin 18	0,030				
CDC42 homolog	0,004				
rho-related GTP-binding protein	0,030				
GAP junction alpha-1 protein / connexin43	0,030				

Proliferation and Apoptosis					
osteoclast stimulating factor	0,000				
FKBP12	0,000				
ets domain protein elk-3	0,004				
CDC42	0.004				
SCGF-beta	0,004				
PIG7	0,007				
interferon gamma receptor	0.009				
high mobility group protein (HMG-I)	0,018				
E2F1	0,010				
growth factor receptor-bound protein 2	0,018				
RalB GTP-binding protein	0,018				
fli-1 oncogene	0,022				

Fig. 14 cont.

B Group II

	p Wilcoxon	Neointima	Control	CASMC	Blood
			mean		
Inflammation					
prostaglandin GH synthetase 1	0.001				
superoxide dismutase 2	0,001				
C5a anaphylatoxin receptor	0,001				
lipoprotein-associated coagulation inhibitor	0,003				
heat shock cognate 71-kDa protein	0,008				
heme oxygenase 1	0,009				
RELB	0,019				
PH-20 homolog	0,027				
Others					
estradiol 17 beta-dehydrogenase 1	0,006				
hydroxyacyl-CoA dehydrogenase	0,004				
steroid 5-alpha reductase 1	0,021				
cholesteryl ester hydrolase	0,025				

Fig. 14 cont.

C Group III

	p Wilcoxon	Neointima mean	Control mean	CASMC mean	Blood mean
Adhesion, Cytoskeleton and ECM					
platelet basic protein	0,000				
migration inhibitory factor-related protein 8	0,000				
L-selectin	0,000				
high-affinity interleukin 8 receptor A	0.000				
alpha-1-antitrypsin	0,000				
proline-rich tyrosine kinase 2 (PYK2)	0,001				
CD18 antigen	0,001				
G-protein-coupled receptor HM74	0,001				
selectin P ligand	0,001				
CD11B antigen	0,001				
ICAM3	0,001				
coronin-like protein P57	0,002				
platelet endothelial cell adhesion molecule	0,009				
matrix metalloproteinase 9	0,012				
CXCR4	0,012				
ninjurin 1	0.032				
integrin beta 7	0,015				

Proliferation and Apoptosis					
c-src kinase	0,000				
DNAX activation protein 12	0,000				
ephrin A receptor 4	0,000				
TRAIL receptor 3	0,001				
ribosomal protein S6 kinase II alpha 1 (RSK1)	0,004				
phospholipase C beta 2	0,006				
BCL-2 binding athanogene-1 (BAG-1)	0,007				
vav oncogene	0,012				
APO-2 ligand	0.012				
BCL-2-related protein A1	0,012				
80-kDa nuclear cap-binding protein	0,015				
interferon regulatory factor 7	0,015				
gamma interferon inducible protein IP30	0.015				
signaling inositol polyphosphate 5 phosphatase	0.033				
activator 1 140-kDa subunit	0.035				
pim-1 proto-oncogene	0,016				

Fig. 14 cont.

C Group III

	p Wilcoxon	Neointima mean	Control mean	CASMC mean	Blood mean
Inflammation					
GM-CSF receptor alpha	0,000				
HLA class II histocompatibility antigen alpha	0,000				
lymphotoxin-beta	0,000				
interleukin-2 receptor gamma	0,000				
leukocyte IgG receptor	0,000				
low affinity immunoglobulin gamma FC receptor II-A	0,000				
myeloid cell nuclear differentiation antigen	0,000				
heat shock 70-kDa protein 6	0,001				
allograft inflammatory factor 1	0,001				
granulocyte colony stimulating factor receptor	0,001				
protein-tyrosine phosphatase 1C	0,001				
C-fgr proto-oncogene	0,001				
lymphocyte antigen	0,001				
rho-GAP hematopoietic protein C1	0,001				
FC-epsilon-receptor gamma	0,001				
CD3	0,001				
lymphokine LAG2	0,001				
p47-PHOX	0,002				
cytidine deaminase	0,002				
proto-oncogene tyrosine-protein kinase lck	0,004				
platelet activating factor receptor	0,004				
macrophage colony stimulating factor 1 receptor	0.004				
tyrosine-protein kinase lyn	0,012				
FMLP-related receptor I	0,012				
interleukin-16	0,012				
interleukin-1 receptor type II	0,012				
lymphoid restricted homolog of SP100 protein	0.005				
IgG receptor FC large subunit P51	0,019				
Others					
clone 23815	0,001				
brain glucose transporter 3	0,004				
hormone-sensitive lipase	0,016				
IMP-dehydrogenase 1	0.027				

Fig. 14 cont.

D Group IV

	p Wilcoxon	Neointima / Control / CASMC / Blood mean
Adhesion, Cytoskeleton and ECM		
MUC18	0,000	
integrin alpha 7B	0,000	
collagen 16 alpha 1	0,000	
tenascin	0,002	
collagen 6 alpha 1	0,004	
desmin	0,000	
peripheral myelin protein 22	0,001	
cytokeratin 6A	0,002	
dual-specificity A-kinase anchoring protein 1	0,003	
homeobox protein HOXB7	0,003	
myotonin-protein kinase	0,008	
microtubule-associated protein 1B	0,014	
collagen 18 alpha 1	0,005	
S100 calcium-binding protein A1	0,001	
integrin alpha 8 (ITGA8)	0,005	
P2X purinoceptor 1	0,007	
cell adhesion kinase	0,005	
integrin alpha 3	0,021	
Inflammation		
alpha-2-macroglobulin	0,001	
extracellular superoxide dismutase	0,001	
inter-alpha-trypsin inhibitor heavy chain H4	0,007	
Others		
adenylate kinase isoenzyme 1	0,025	
carboxypeptidase H	0,018	
autosomal dominant polycystic kidney disease II	0,000	
PC8 convertase	0,000	
adipocyte fatty acid-binding protein 4	0,001	
brain-specific polypeptide PEP-19	0,004	

Fig. 14 cont.

D Group IV

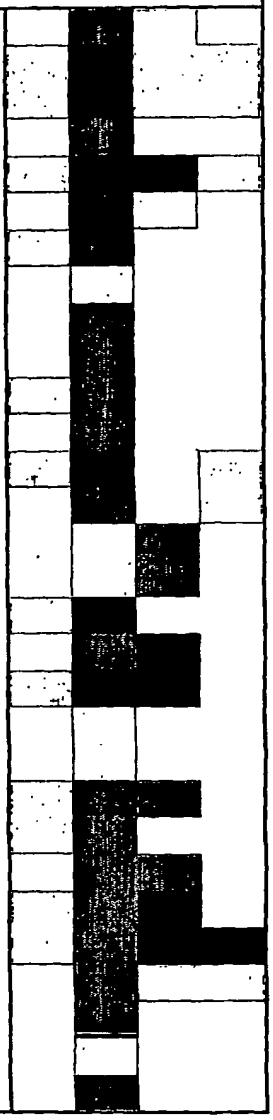
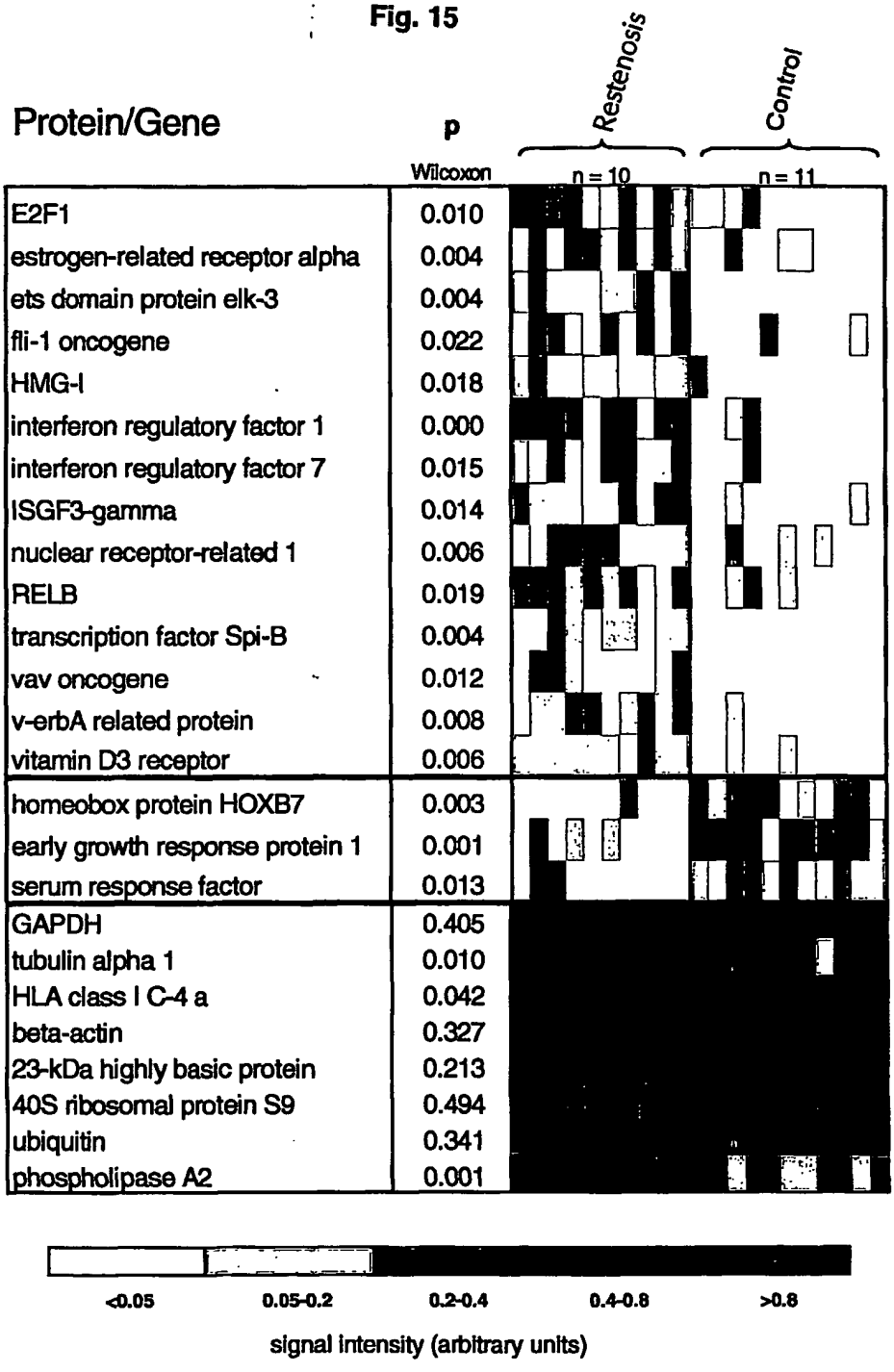
Proliferation and Apoptosis	p Wilcoxon mean	Neointima Control CASMC Blood
neurotrophic tyrosine kinase receptor-related 3	0,000	
insulin-like growth factor binding protein 6	0,001	
early growth response protein 1	0,001	
tuberin	0,001	
metallothionein-III	0,001	
GADD45 beta	0,002	
collagen 18 alpha 1 subunit (COL18A1)	0.021	
NT-3 growth factor receptor	0,003	
RAD1	0,003	
frizzled-related FrzB FRITZ	0,003	
insulin-like growth factor 1 receptor (IGF1R)	0.021	
G1/S-specific cyclin D1	0,003	
c-fos proto-oncogene	0,003	
early response protein NAK1	0,004	
neurogenic locus notch protein	0,005	
RYK	0,005	
mammary-derived growth inhibitor	0,007	
CBL-B	0,007	
cyclin-dependent kinase inhibitor 1	0,009	
high-affinity nerve growth factor receptor	0,011	
purine-rich single-stranded DNA-binding prot. alpha	0,011	
p16-INK4	0,011	
serum response factor	0,013	
NuMA	0,014	
guanine nucleotide-binding protein G(Y) alpha 11	0,015	
BIGH3	0,015	
VEGF B + VRF186	0,016	
insulin-like growth factor I receptor	0,017	
P126	0,023	
GADD45 gamma	0,023	

Fig. 15


Protein/Gene
p
Wilcoxon
Restenosis
Control
n = 10
n = 11
E2F1 0.010
estrogen-related receptor alpha 0.004
ets domain protein elk-3 0.004
fli-1 oncogene 0.022
HMG-I 0.018
interferon regulatory factor 1 0.000
interferon regulatory factor 7 0.015
ISGF3-gamma 0.014
nuclear receptor-related 1 0.006
RELB 0.019
transcription factor Spi-B 0.004
vav oncogene 0.012
v-erbA related protein 0.008
vitamin D3 receptor 0.006
homeobox protein HOXB7 0.003
early growth response protein 1 0.001
serum response factor 0.013
GAPDH 0.405
tubulin alpha 1 0.010
HLA class I C-4 a 0.042
beta-actin 0.327
23-kDa highly basic protein 0.213
40S ribosomal protein S9 0.494
ubiquitin 0.341
phospholipase A2 0.001
<0.05
0.05-0.2
0.2-0.4
0.4-0.8
>0.8
signal intensity (arbitrary units)

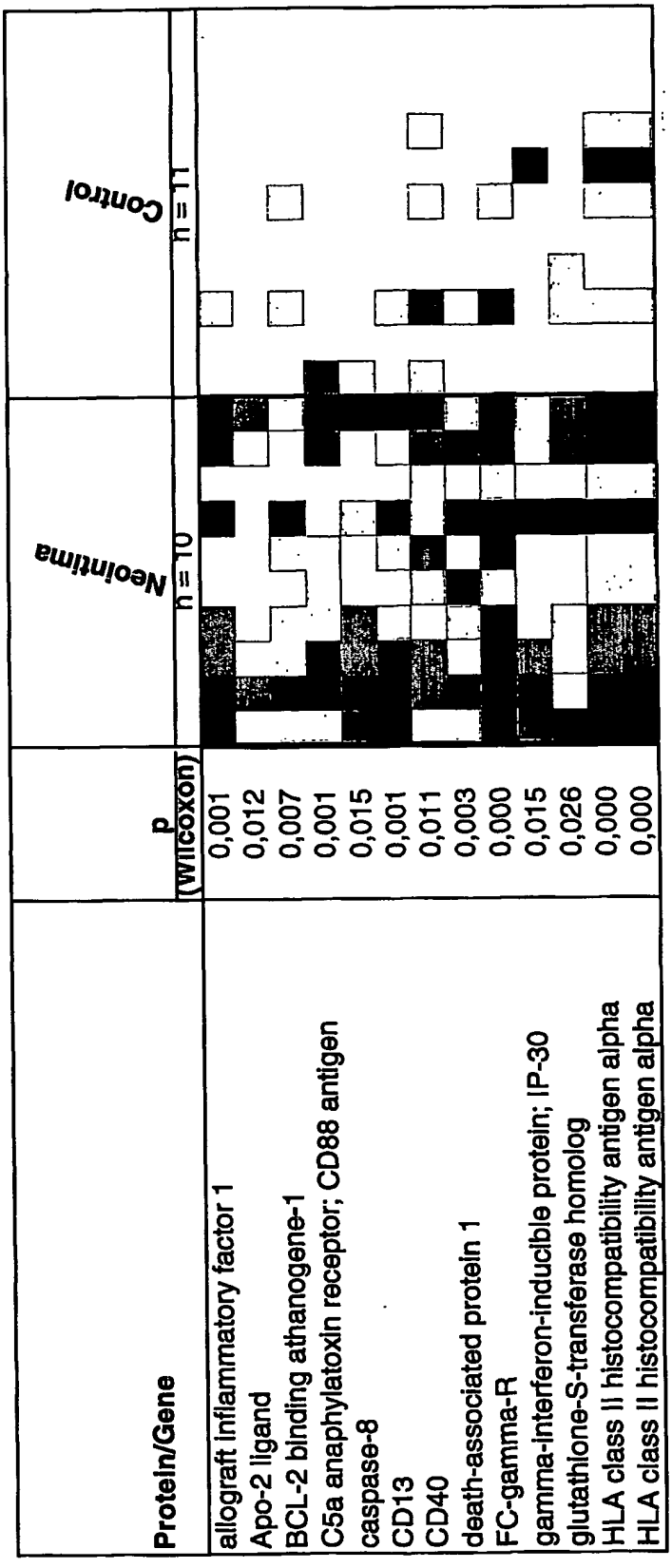

Protein/Gene
p (Wilcoxon)
Neointima n = 10
Control n = 11
allograft inflammatory factor 1 0,001
Apo-2 ligand 0,012
BCL-2 binding athanogene-1 0,007
C5a anaphylatoxin receptor; CD88 antigen 0,001
caspase-8 0,015
CD13 0,001
CD40 0,011
death-associated protein 1 0,003
FC-gamma-R 0,000
gamma-interferon-inducible protein; IP-30 0,015
glutathione-S-transferase homolog 0,026
HLA class II histocompatibility antigen alpha 0,000
HLA class II histocompatibility antigen alpha 0,000

Fig. 16

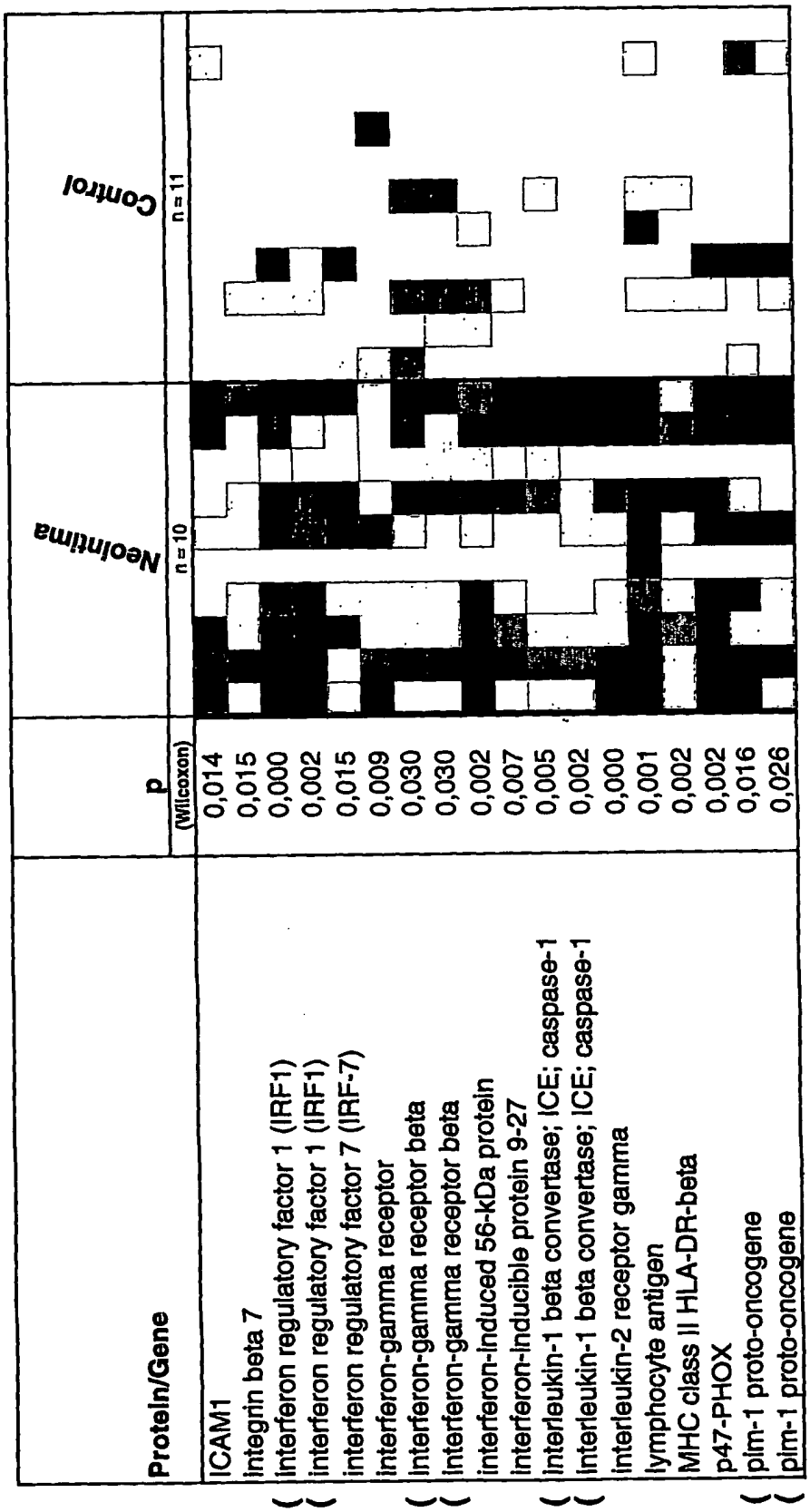

Protein/Gene
p (Wilcoxon)
Neointima n = 10
Control n = 11
ICAM1 0,014
integrin beta 7 0,015
interferon regulatory factor 1 (IRF1) 0,000
interferon regulatory factor 1 (IRF1) 0,002
interferon regulatory factor 7 (IRF-7) 0,015
interferon-gamma receptor 0,009
interferon-gamma receptor beta 0,030
interferon-gamma receptor beta 0,030
interferon-induced 56-kDa protein 0,002
interferon-inducible protein 9-27 0,007
interleukin-1 beta convertase; ICE; caspase-1 0,005
interleukin-1 beta convertase; ICE; caspase-1 0,002
interleukin-2 receptor gamma 0,000
lymphocyte antigen 0,001
MHC class II HLA-DR-beta 0,002
p47-PHOX 0,002
pim-1 proto-oncogene 0,016
pim-1 proto-oncogene 0,026

Fig. 16 cont.

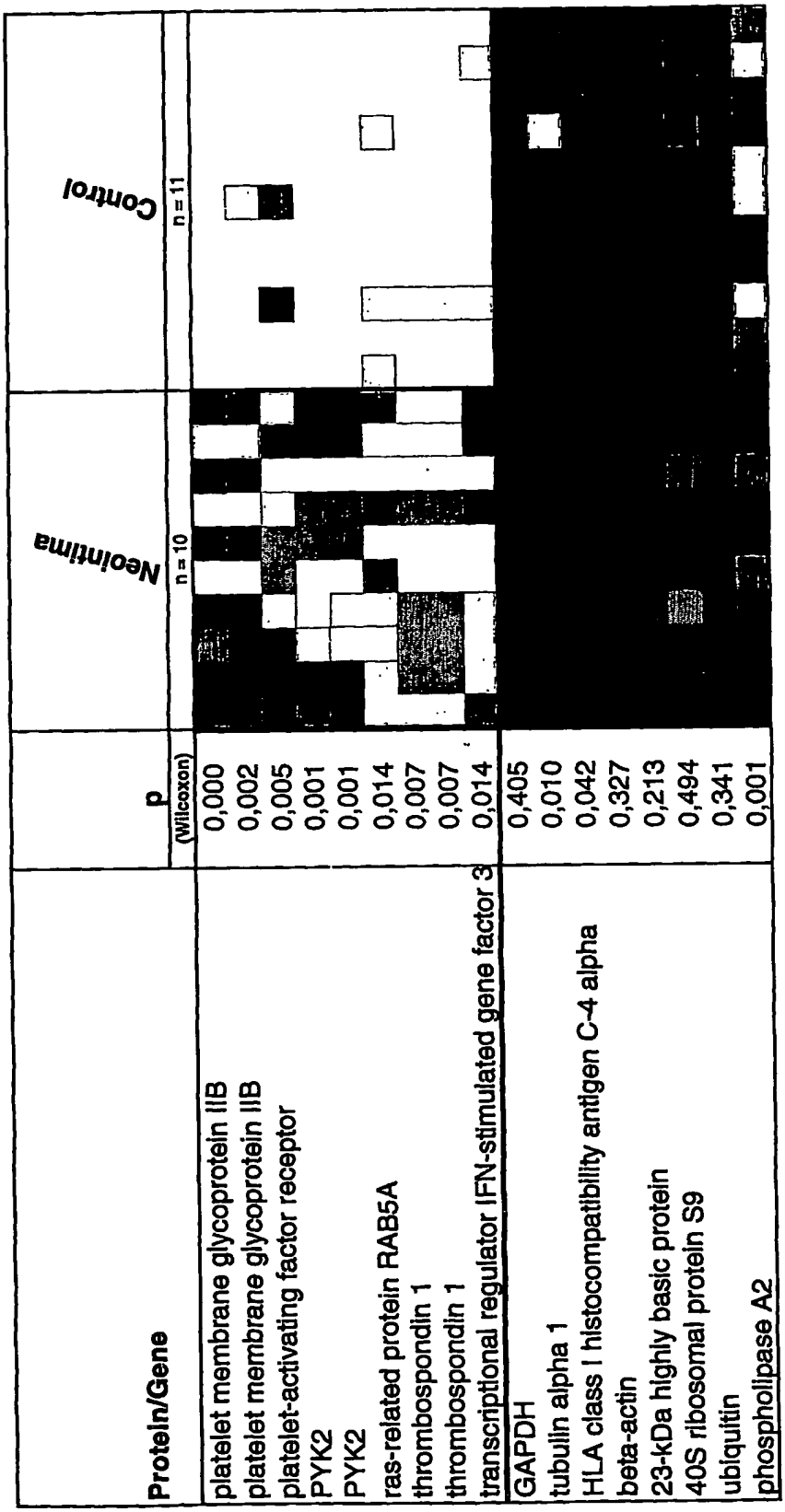

Protein/Gene
p (Wilcoxon)
Neointima n = 10
Control n = 11
platelet membrane glycoprotein IIB 0,000
platelet membrane glycoprotein IIB 0,002
platelet-activating factor receptor 0,005
PYK2 0,001
PYK2 0,001
ras-related protein RAB5A 0,014
thrombospondin 1 0,007
thrombospondin 1 0,007
transcriptional regulator IFN-stimulated gene factor 3 0,014
GAPDH 0,405
tubulin alpha 1 0,010
HLA class I histocompatibility antigen C-4 alpha 0,042
beta-actin 0,327
23-kDa highly basic protein 0,213
40S ribosomal protein S9 0,494
ubiquitin 0,341
phospholipase A2 0,001

Fig. 16 cont.

Fig. 17

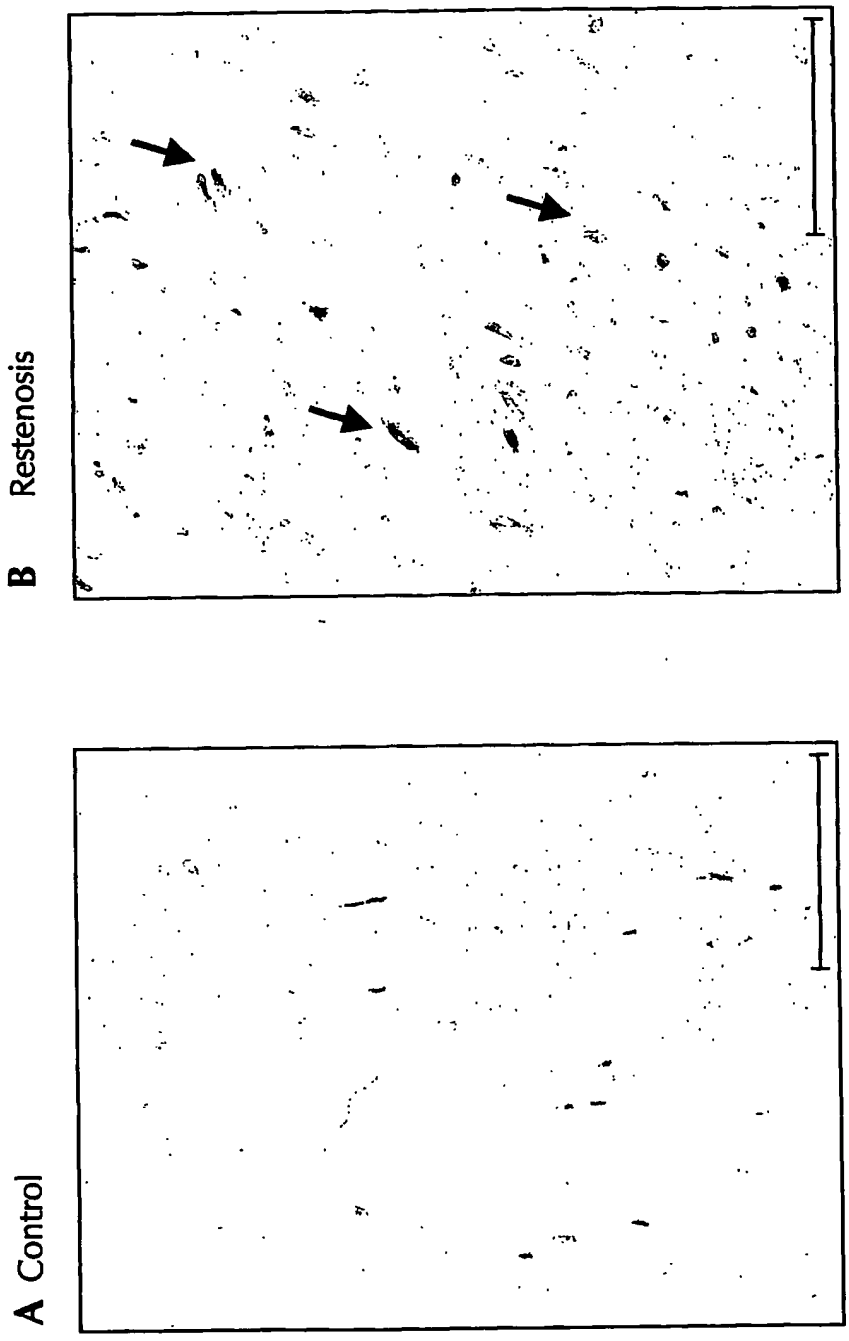


Fig. 18

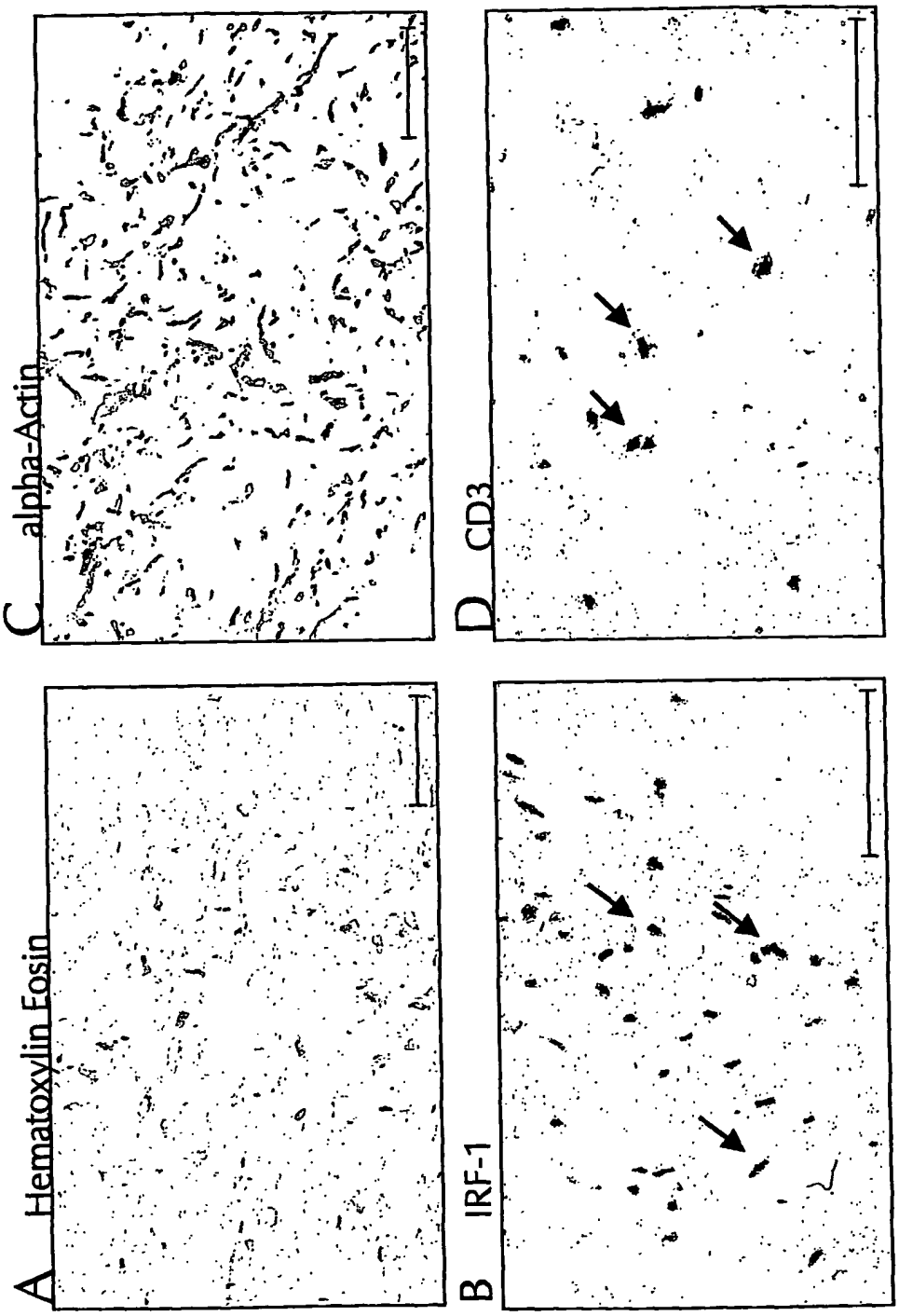


Fig. 19

IFN-γ induced genes	p (Wilcoxon)	Neointima	Control	CASMC	CASMC+IFN
allograft inflammatory factor 1	0,001				
APO-2 ligand	0,012				
BCL-2 binding athanogene-1	0,007				
C5a anaphylatoxin receptor	0,001				
caspase-1	0,005				
caspase-8	0,015				
death-associated protein 1	0,003				
FC-gamma-R	0,000				
gamma-interferon-inducible protein IP	0,015				
glutathione-S-transferase	0,026				
HLA class II alpha	0,000				
ICAM-1	0,014				
integrin alpha 2B	0,000				
integrin beta 7	0,015				
interferon-gamma receptor	0,009				
interferon-gamma receptor beta	0,030				
interferon-induced 56-kDa protein	0,002				
interferon-inducible protein 9-27	0,007				
interleukin-2 receptor gamma	0,000				
interferon regulatory factor-1	0,000				
interferon regulatory factor-7	0,015				
ISGF3-γ	0,014				
lymphocyte antigen	0,001				
MHC class II HLA-DR-beta	0,002				
p47-PHOX	0,002				
pim-1 proto-oncogene	0,016				
platelet-activating factor receptor	0,004				
PYK2	0,001				
RAB5A	0,014				
thrombospondin 1	0,007				

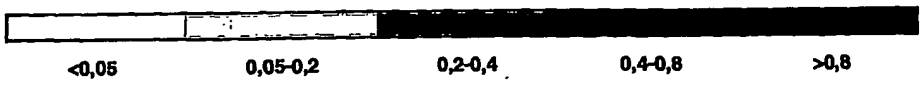


Fig. 20

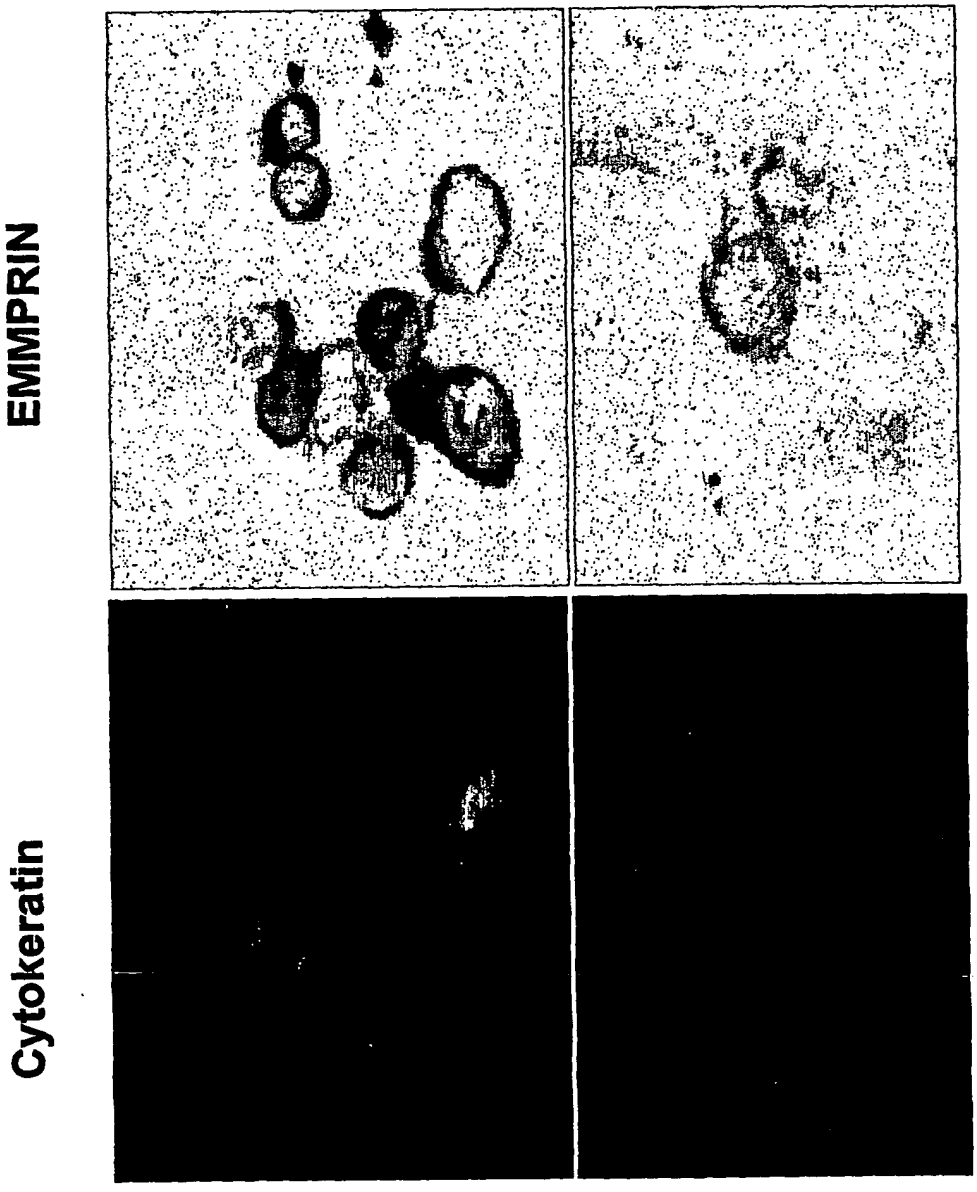


Fig. 21

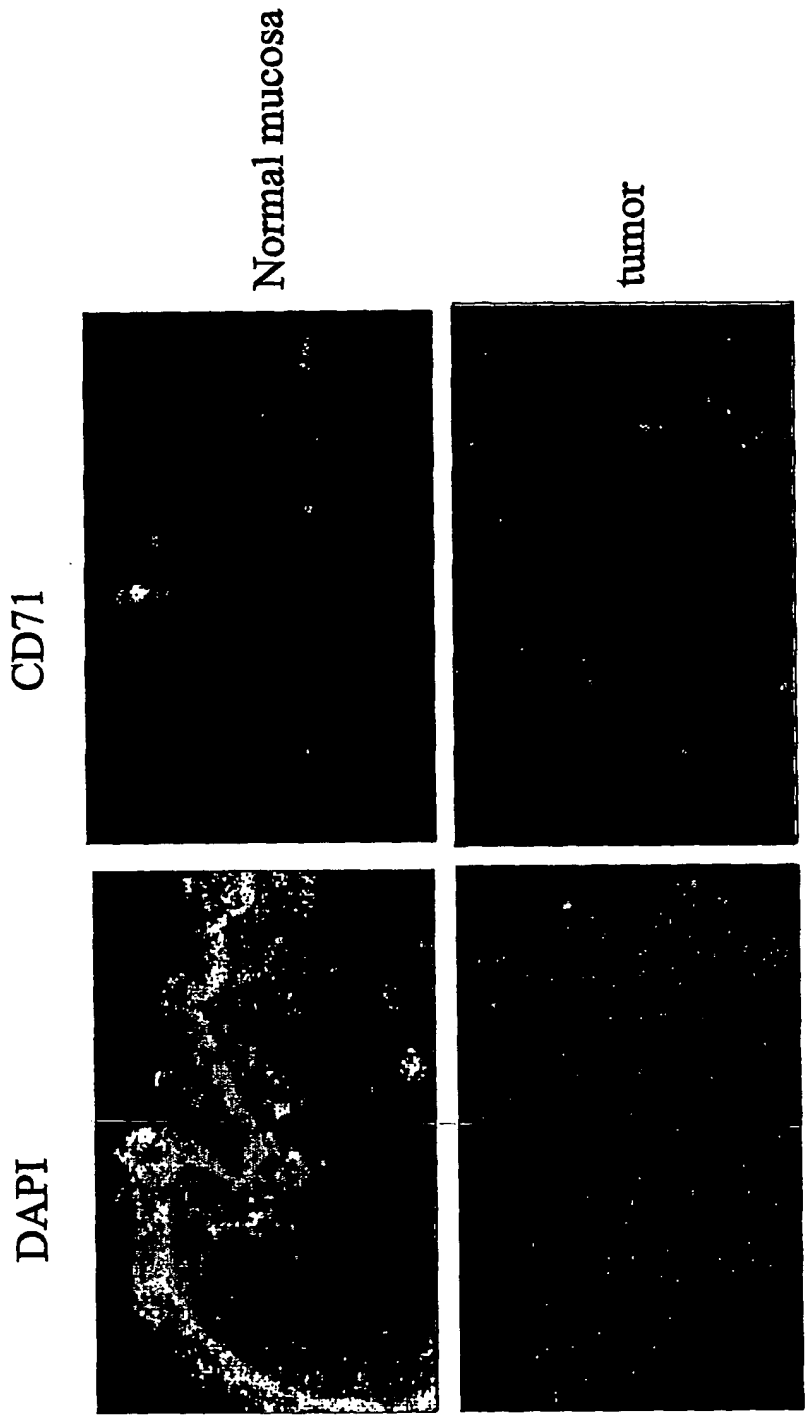


Fig. 22

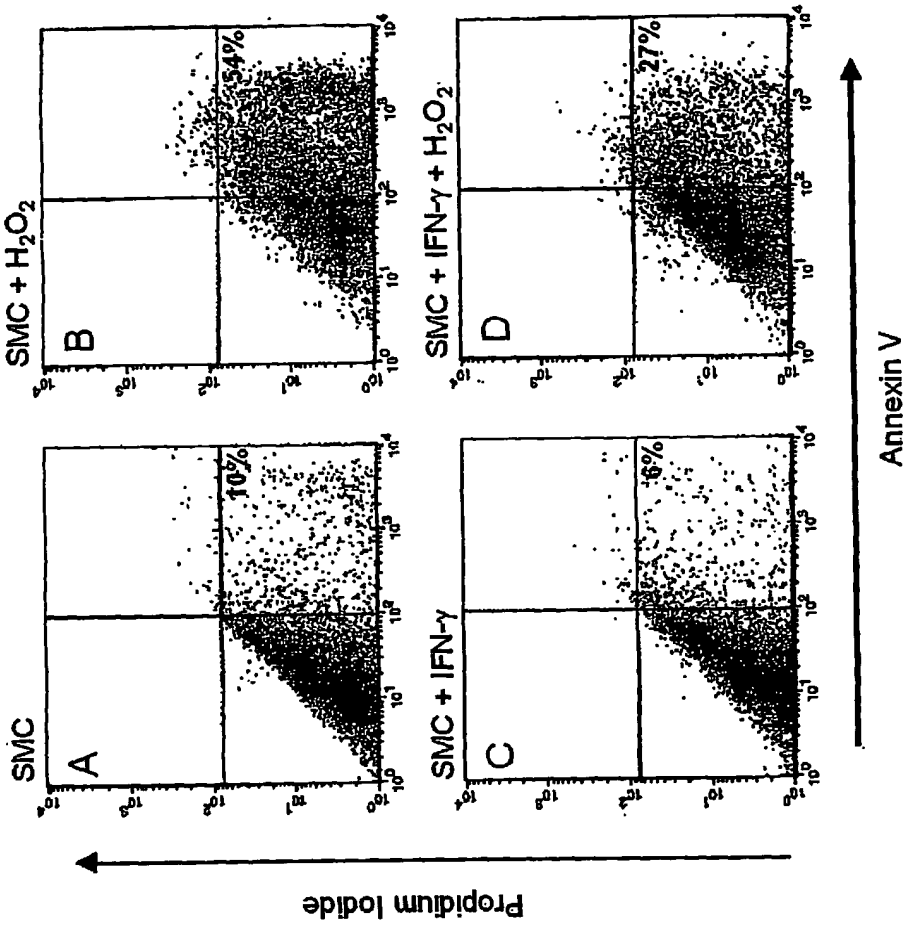
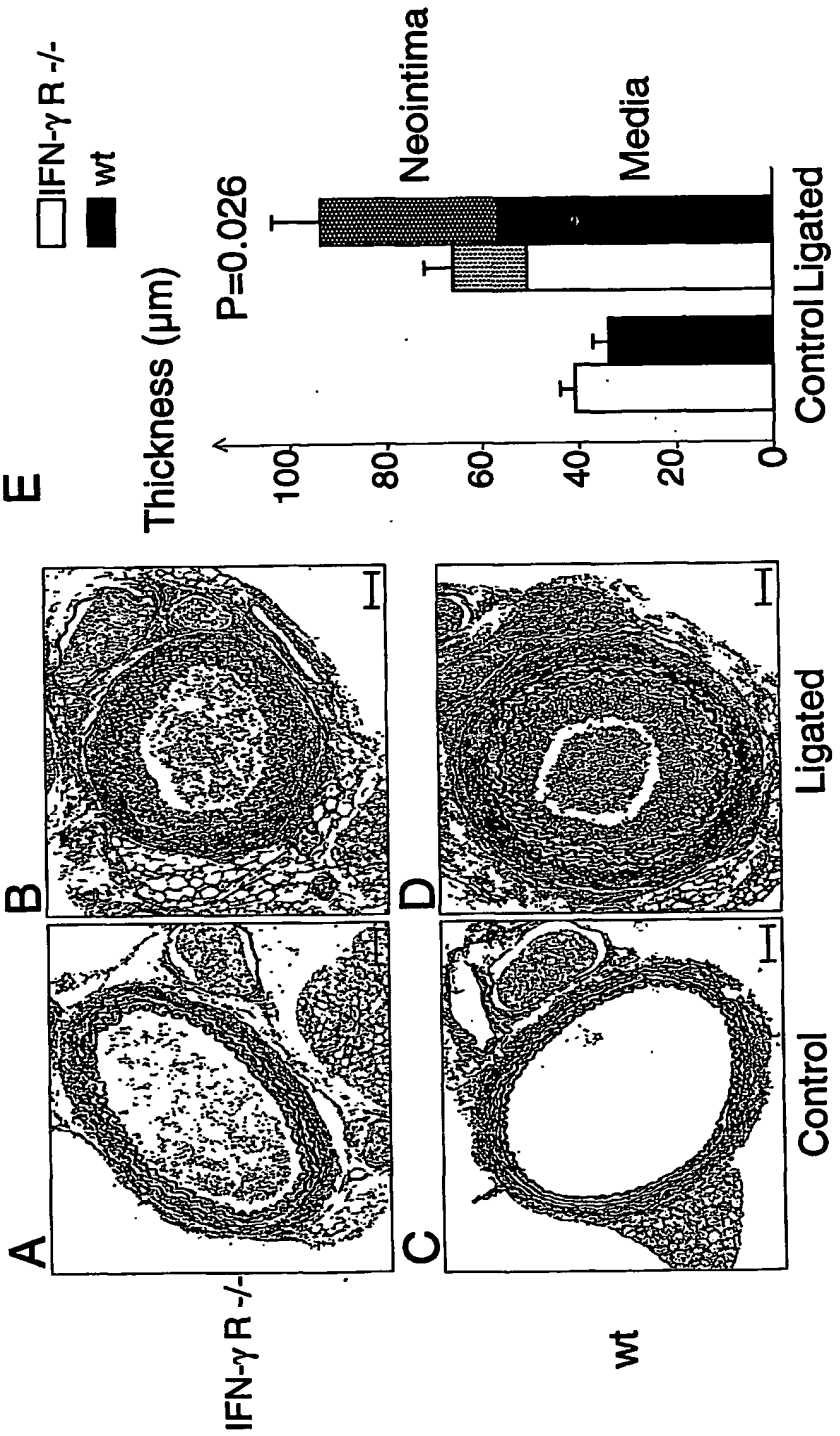

SMC + H_2O_2
B
54%
SMC + IFN-γ + H_2O_2
D
27%
SMC
A
10%
SMC + IFN-γ
C
6%
Annexin V
Propidium Iodide

Fig. 23


A
B
C
D
E
IFN-γ R -/-
wt
Control
Ligated
Control Ligated
Media
Neointima
P=0.026
Thickness (µm)
100
80
60
40
20
0
IFN-γ R -/-
wt

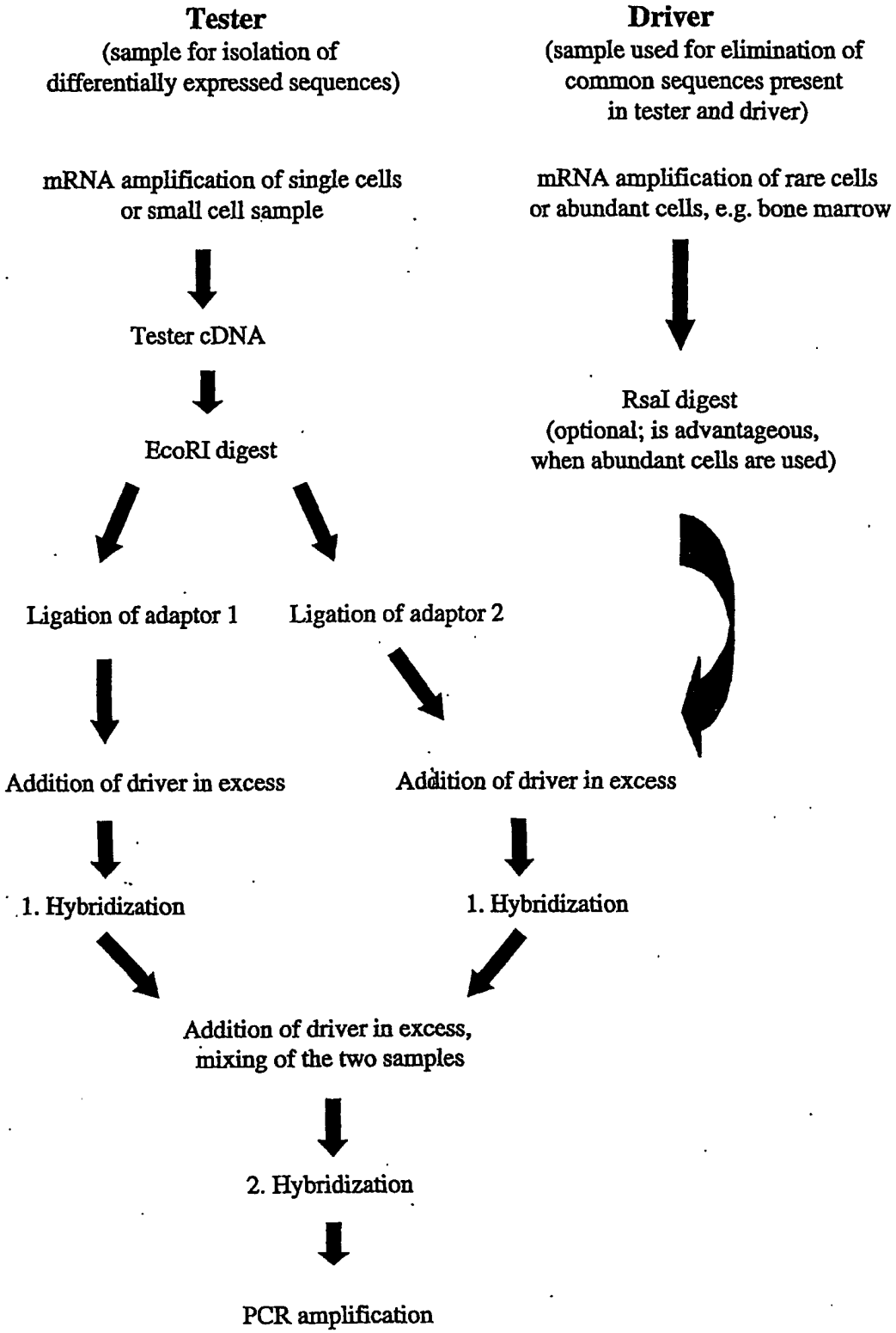

Tester
(sample for isolation of differentially expressed sequences)
Driver
(sample used for elimination of common sequences present in tester and driver)
mRNA amplification of single cells or small cell sample
mRNA amplification of rare cells or abundant cells, e.g. bone marrow
Tester cDNA
RsaI digest
(optional; is advantageous, when abundant cells are used)
EcoRI digest
Ligation of adaptor 1
Ligation of adaptor 2
Addition of driver in excess
Addition of driver in excess
1. Hybridization
1. Hybridization
Addition of driver in excess, mixing of the two samples
2. Hybridization
PCR amplification

Fig. 24

Screening of colonies by southern blot using driver and tester as probe

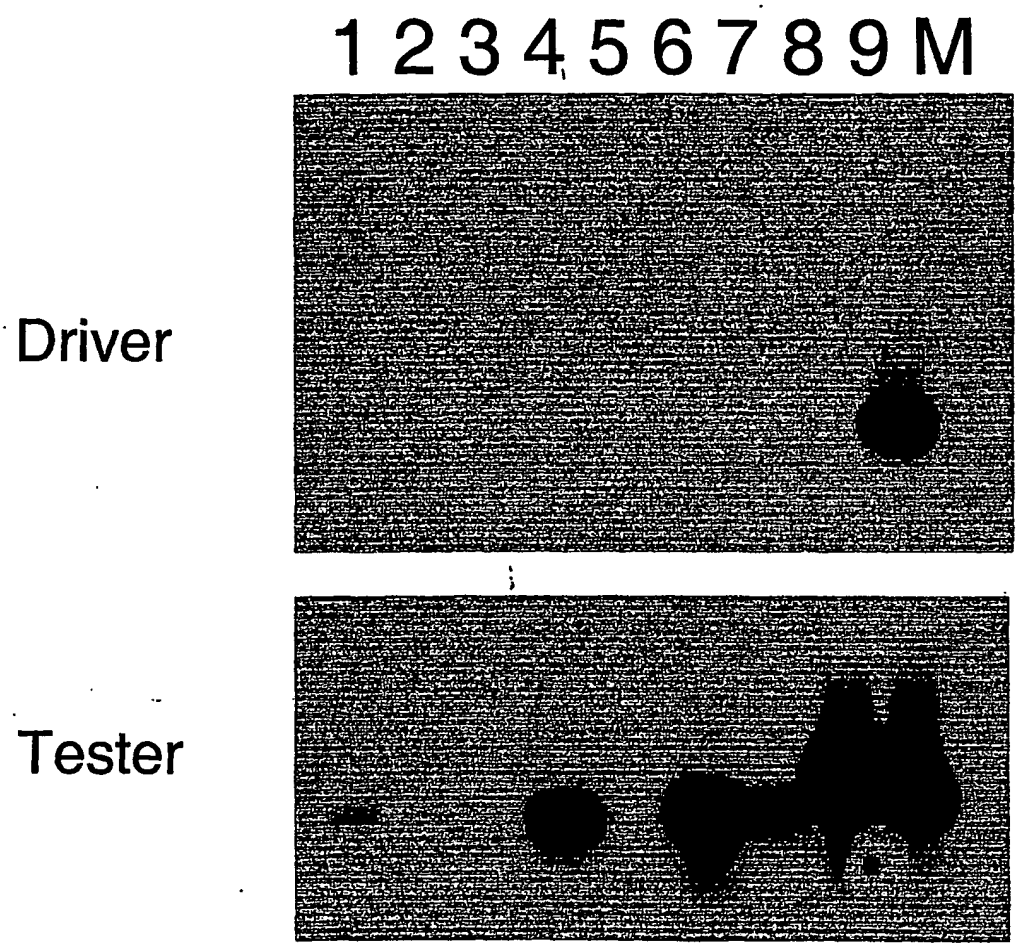
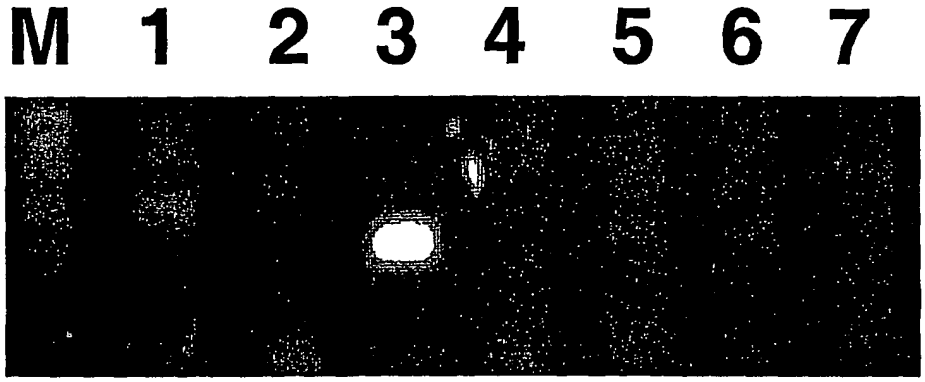


Fig. 25

Fig. 26

Differential expression of ESE1



1 - 4: single breast cancer cells
5-7: bone marrow of healthy donors

SEQUENCE LISTING

<110> Micromet GmbH

<120> mRNA amplification

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